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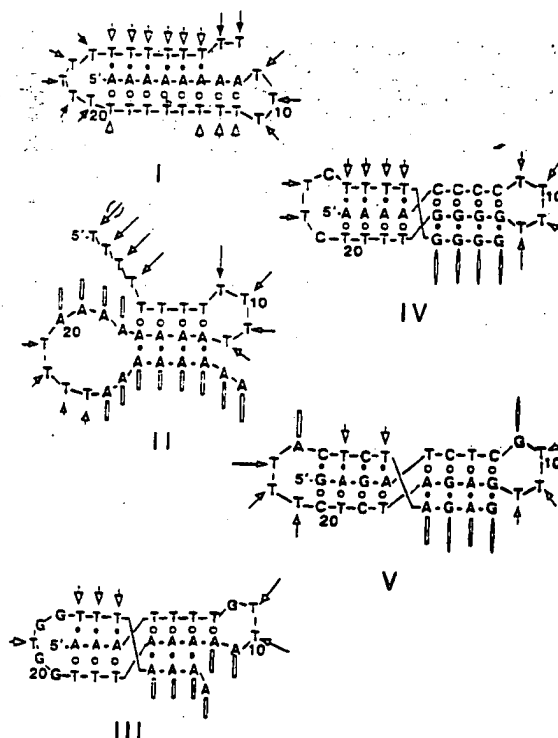
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(54) Title: TRIPLE-HELIX FORMATION AT (PU_NPY_N).(PU_NPY_N) TRACTS

(57) Abstract

An oligonucleotide capable of forming a triple-helix with a nucleic acid molecule containing a target nucleic acid sequence is described, where the oligonucleotide is comprised of tandem, alternating tracts of purine and pyrimidine sequences, and where the oligonucleotide is effective to form a triple-helix structure with a target nucleic acid sequence having alternating tracts of purine and pyrimidine sequences. A method of cleaving the nucleic acid molecule is also described.



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TRIPLE-HELIX FORMATION
AT (PU_NPY_N)•(PU_NPY_N) TRACTS

5

Field of the Invention

The present invention relates to the use of triple helical DNA in the control of gene expression, including therapeutics, and the development of sequence-specific DNA cleaving agents. The present invention describes third strand molecules that utilize both Pu•PuPy and Py•PuPy base triplets to form a continuous DNA triple-helix at tandem oligo-
10 purine (Pu_n) and oligopyrimidine (Py_n) tracts.
15

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Background of the Invention

Much recent research has been focussed on the nucleic acid triple helix, a structure that was discovered more than 30 years ago in synthetic poly-
5 ribonucleotides consisting exclusively of purine and pyrimidine strands (Felsenfeld et al., 1957). Triplex DNA has been shown to form in (i) oligo-purine-oligopyrimidine mirror repeats under superhelical stress (H-DNA) (Lyamichev et al., 1986; Mirkin
10 et al., 1987; Voloshin et al., 1988; Htun & Dahlberg, 1988; Kohwi & Kohwi-Shigematsu, 1988; Hanvey et al., 1988; Wells et al., 1988; Johnston, 1988; Glover & Pulleyblank, 1990), (ii) between linear single strands and native duplex DNA (intermolecular
15 triplexes) (Moser & Dervan, 1987; Francois et al., 1988; Pilch et al., 1990), and (iii) within linear single strands (intramolecular triplexes) (Haner & Dervan, 1990; Chen, 1991) under suitable conditions.

Although a detailed X-ray crystal structure of
20 a triple helix is still lacking, a substantial body of chemical and physical evidence indicates that recognition of a duplex oligopurine-oligopyrimidine tract by an oligopyrimidine single strand [Figure 1a(i)] involves the occupation of the major groove
25 of the duplex by the oligopyrimidine strand and the formation of the PyPuPy base triplets TAT and C⁺GC via Hoogsteen hydrogen bonding (Felsenfeld et al., 1957; Morgan & Wells, 1968; Lee et al., 1979; Praseuth et al., 1988; Johnston, 1988; Rajagopal &
30 Feigon, 1989; de los Santos et al., 1989). The oligopyrimidine strand lies in parallel orientation to the oligopurine tract of the duplex (Moser & Dervan 1987; Praseuth et al., 1988), and the pairing of T with AT and C⁺ with GC makes the recognition
35 sequence-specific. Because the C⁺GC triplet requires protonated cytosines on the Hoogsteen (third)

strand, triplex formation is facilitated by low pH (Mirkin et al., 1987).

In addition to PyPuPy triplets, another general class of base triplets, PuPuPy (G:GC and A:AT), has also been proposed to explain the sequence-specific recognition of oligopurine-oligopyrimidine tracts in duplex DNA by oligopurine single strands (Figure 1a(ii)). In this case the oligopurine single strand, which again lies in the major groove, is antiparallel to the oligopurine tract in duplex DNA (Beal & Dervan, 1991). Triplexes of the PuPuPy type can form independently of pH (Kohwi & Kohwi-Shigematsu, 1988; Kohwi-Shigematsu & Kohwi, 1991) and thus are potentially more useful in many applications than triplexes of the PyPuPy type.

In addition to the above base triplets, unusual triplets such as GTA (Griffin & Dervan, 1989; Radhakrishnan et al., 1991), C⁺(or C):AT, and UGC (Pei et al., 1991) and unnatural triplets such as I:AT and I:GC (Letai et al., 1988) have also been documented in triple helical nucleic acids.

Typically, DNA recognition via triplex formation has been restricted to oligopurine-oligopyrimidine tracts using single strands consisting exclusively of either purines or pyrimidines. The following approaches (Horne & Dervan, 1990) have been proposed for recognition of all four bases in triplex formation: (1) synthesizing novel, unnatural bases to complete the triplet code; (2) excluding the recognition of certain base pairs in a triplex motif by incorporating abasic sites in the single strand, which may decrease the specificity of triplex formation; and (3) designing homopyrimidine single strands capable of binding to alternate strands of duplex DNA. In the last approach, polarity considerations required the design of an oligonucleotide with an unnatural 3'-3' linkage

having the proper spacing between two oligopyrimidine strings (Horne & Dervan, 1990).

Experiments performed in support of the present invention suggest a different approach to achieve
5 recognition of all four bases in triplex formation. This approach does not require the synthesis of unusual bases or linkages.

Summary of the Invention

10 An oligonucleotide capable of forming a triple-helix with a nucleic acid molecule containing a target nucleic acid sequence is described, where the oligonucleotide is comprised of tandem, alternating
15 tracts of purine and pyrimidine sequences, and where the oligonucleotide is effective to form a triple-helix structure with a target nucleic acid sequence having alternating tracts of purine and pyrimidine sequences.

The oligonucleotide of the present invention
20 typically contain at least one purine tract adjacent at least one pyrimidine tract. Also, the target sequence, which oligonucleotide binding is directed toward, usually contains at least about 8 nucleotides.

25 The nucleic acid target sequences of the present invention can be duplex DNA molecules, single-strand DNA molecules, and/or RNA molecules. Typically, when the nucleic acid molecule is a single stranded nucleic acid molecule, and the oligonucleotide contributes two of the three strands of
30 the triple-helix.

In one embodiment of the present invention, the oligonucleotide further includes at least one moiety attached to the oligonucleotide, where the moiety is
35 capable of cleaving the DNA molecule containing the target nucleic acid sequence. A representative group of such cleaving moieties includes the follow-

ing: phenanthroline Cu(II), Zn(II), Fe(II)-EDTA, Cu(II)-bipyridine, and Cu(II)-terpyridine.

Also described is a method of cleaving a nucleic acid molecule containing a target nucleic acid sequence, wherein the nucleic acid molecule is contacted with an oligonucleotide comprised of tandem, alternating tracts of purine and pyrimidine sequences, where the oligonucleotide is effective to form a triple-helix structure with a target nucleic acid sequence having alternating tracts of purine and pyrimidine sequences, and where the oligonucleotide includes at least one moiety which is capable of cleaving the nucleic acid molecule containing the target nucleic acid sequence. In this method, the oligonucleotide concentration is effective to produce cleavage of the nucleic acid molecule containing the target nucleic acid sequence

The target nucleic acid can be single- or double- stranded; this includes DNA and RNA targets.

Brief Description of the Figures

Figure 1. A. Schematic illustration of different triplex motifs. (i) A PyPuPy triple helix in which the third, oligopyrimidine strand is parallel to the purine strand of the duplex. (ii) A PuPuPy triple helix in which the third, oligopurine strand is antiparallel to the purine strand of the duplex. (iii) A triplex spanning tandem oligopurine and oligopyrimidine tracts in which the purine block of the third strand forms hydrogen bonds with purines of one strand of the duplex (via PuPuPy base triplets), whereas the pyrimidine block of the third strand hydrogen bonds to the purine tract of the other strand of the duplex via PyPuPy base triplets. Within this hybrid triplex, the required polarity of the third strand is maintained. The polarity of each strand is shown by a half-ar-

row. Open circles represent Watson-Crick hydrogen bonding and closed circles represent Hoogsteen or another type of hydrogen bonding.

B. A ribbon model for an intramolecular
5 triplex of type iii, in which the third strand recognizes alternate strands of a hairpin duplex. Here the third-strand region (shown in the middle in gray and black-and-white stripes) folds back on the hairpin duplex (white ribbon), forming two loops
10 (top and bottom). The purine tract of the third strand (gray) forms base pairs (black bars) with the purine tract of one strand of the Watson-Crick hairpin (making PuPuPy base triplets), whereas the
15 pyrimidine tract of the third strand (black-and-white) forms Hoogsteen base pairs (banded bars) with the purine tract of the other Watson-Crick strand making PyPuPy base triplets. White bars represent Watson-Crick hydrogen bonding, and arrows indicate the polarity (5' to 3') of the strand.

20 Figure 2. This figure illustrates the KMnO_4 reactivity of oligonucleotide I (SEQ ID NO:1) (Figure 8).

Figure 3. This figure illustrates the KMnO_4 and
DEPC reactivity of oligonucleotide II (SEQ ID NO:2)
25 (Figure 8).

Figure 4. This figure illustrates the KMnO_4 and
DEPC reactivity of oligonucleotide III (SEQ ID NO:3)
(Figure 8).

Figure 5. This figure illustrates the KMnO_4 and
30 DEPC reactivity of oligonucleotide IV (SEQ ID NO:4) (Figure 8).

Figure 6. This figure illustrates the KMnO_4 ,
DEPC, and DMS reactivity of oligonucleotide V (SEQ ID
NO:5) (Figure 8).

35 Figure 7. This figure summarizes the reactivity patterns and deduced secondary structures for each of oligonucleotides I-V: Closed arrows, KMnO_4

reactivity that is not sensitive to Mg^{++} ions; the size of the arrow roughly correlates with the extent of base reactivity. (The arrow shown in parentheses is a presumably reactive thymine that was run off the gel.) Open arrows, $KMnO_4$ reactivity of thymines sensitive to the presence of Mg^{++} ions. Open rectangles, DEPC reactivity of adenines, with the symbol size roughly correlating with the extent of reactivity. Open cigar-shaped symbols, DMS reactivity of guanines. Lines connecting bases show the continuity of the backbone. Open circles, Watson-Crick hydrogen bonding. Closed circles, Hoogsteen or PuPu-type hydrogen bonding.

Figure 8. This figure presents the nucleotide sequences of oligonucleotides I-V.

Figure 9 (A-C) illustrates three potential sites for targeting within the LTR region of HIV-1 with single-stranded oligonucleotides.

Figure 10 presents the sequences of oligonucleotides A1 (SEQ ID NO:9) and A2 (SEQ ID NO:10), B1 (SEQ ID NO:11) and B2 (SEQ ID NO:12), C1 (SEQ ID NO:13) and C2 (SEQ ID NO:14) which are designed to target sequences of Figure 9A, 9B, and 9C, respectively. Next to each of these oligonucleotides is the general pattern of base triplets expected to form when triplexes are formed. Oligonucleotides K (SEQ ID NO:15), L (SEQ ID NO:16), and M (SEQ ID NO:17) are oligonucleotides used in control experiments.

Figure 11 illustrates the chemistry of the attachment of a phenanthroline moiety to a cysteine-containing polypeptide.

Figure 12A illustrates three TDA target sites (D, SEQ ID NO:18; E, SEQ ID NO:19; F, SEQ ID NO:20) within the 5786-8887 nucleotide region of the HIV-1 genome. Figure 12B shows a schematic representation of triplex formation at target mRNA sequences D, E,

and F. For each mRNA sequence two oligonucleotides (1 and 2) are shown: D1 (SEQ ID NO:21), D2 (SEQ ID NO:22); E1 (SEQ ID NO:23), E2 (SEQ ID NO:24); and F1 (SEQ ID NO:25), F2 (SEQ ID NO:26). Oligonucleotides D_x (SEQ ID NO:27), E_x (SEQ ID NO:28), and F_x (SEQ ID NO:29) are the complementary oligonucleotides designed to function as conventional antisense agents. X indicate abasic sites of oligonucleotides -- in the above listed SEQ ID NOs., the abasic site is indicated by an N.

Figure 13 provides an overview of a method for targeting and inactivation of HIV mRNA using TDA oligonucleotides.

Figure 14A shows oligonucleotide sequences containing the target sequences for Example 4B. These sequences are also presented as: Target sequence #1, SEQ ID NO:30 and SEQ ID NO:31; Target sequence #2, SEQ ID NO:32 and SEQ ID NO:33; Target sequence #3, SEQ ID NO:34 and SEQ ID NO:35; Target sequence #4, SEQ ID NO:36 and SEQ ID NO:37; Target sequence #5, SEQ ID NO:38 and SEQ ID NO:39; Target sequence #6, SEQ ID NO:40 and SEQ ID NO:41; Target sequence #7, SEQ ID NO:42 and SEQ ID NO:43; and Target sequence #8, SEQ ID NO:44 and SEQ ID NO:45.

Figure 14B shows two oligonucleotides of the present invention which can form triplexes with Target sequence #3: SEQ ID NO:46 and SEQ ID NO:47.

Figure 15A shows oligonucleotides containing unusual bases for use in triplex formation: I1, SEQ ID NO:49; I2, SEQ ID NO:50; G1, SEQ ID NO:51; and A1, SEQ ID NO:52. Figure 15B presents two exemplary target sequences for oligonucleotide I1 (SEQ ID NO:48 and SEQ ID NO:53).

Detailed Description of the Invention

I. Intramolecular Triple-Helix Formation.

Oligonucleotides were synthesized which were designed to fold into intramolecular triplexes as shown schematically in Figure 7. Analysis of these oligonucleotides using several different chemical probes indicated that triple-helix formation occurs at tandem oligopurine and oligopyrimidine tracts. This finding suggests that designing oligonucleotides for triplex binding, so as to utilize both known types of base triplets together with strand switching, can significantly relax the requirement of homopurine sequences for triplex formation.

The following chemical reagents have been used in the analysis of triplex formation using the oligonucleotides of the present invention.

Potassium permanganate is sensitive to the secondary structure of nucleic acids, and it specifically reacts with exposed thymines at the 5-6 double bond (Kochetkov & Budovskii, 1972). Thus thymines in a single-stranded DNA are sensitive to KMnO_4 modification, but become resistant when involved in a duplex (Hayatsu & Ukita, 1967). Haner & Dervan (1991) have demonstrated that thymines on a single strand become less reactive toward KMnO_4 when the single strand is incorporated into a triple helix by occupying the major groove of a DNA duplex.

DMS reacts predominantly at the N-7 position of guanines (Maxam & Gilbert, 1980). In a triple helix, the N-7 position of guanines in the Watson-Crick duplex is involved in hydrogen bonding to the third strand in both C^+GC and GGC base triplets; in the case of C^+GC triplets, guanines have been shown to become resistant to DMS modification (Johnston, 1988; Hanvey et al., 1988).

DEPC, which carbethoxylates purines ($\text{A} > \text{G}$) primarily at the N-7 position, is useful for probing

adenine residues involved in triplex formation. In T-AT base triplets, adenines in the Watson-Crick strand show protection from DEPC modification because of the Hoogsteen hydrogen bonding involving the N-7 position (Johnston, 1988; Htun & Dahlberg, 1988; Hanvey et al., 1988). Similar protection is expected for the Watson-Crick adenine of the A-AT base triplet.

Oligonucleotide I (Figure 8) has the general form $(\text{Pu})_n\text{N}_4(\text{Py})_n\text{N}_4(\text{Py})_n$ and is expected to form an intramolecular triple helix with Py-Pu-Py base triplets. On the other hand, oligonucleotide II (Figure 8) has the general form of $(\text{Py})_n\text{N}_4(\text{Pu})_n\text{N}_4(\text{Pu})_n$ and thus has a potential of forming an intramolecular triplex with Pu-Pu-Py base triplets.

Oligonucleotides III-V (Figure 8) consist of two different triplex-forming motifs fused together, i.e., these oligonucleotides can be considered as hybrids of oligonucleotides I and II. They belong to the general form $(\text{Pu})_n(\text{Py})_n\text{N}_4(\text{Pu})_n(\text{Py})_n\text{N}_4(\text{Py})_n(\text{Pu})_n$ and can potentially form a triplex consisting of a block of Py-Pu-Py base triplets connected to a block of Pu-Pu-Py base triplets. For clarity, each oligonucleotide is described in three segments: 5', middle, and 3' segments.

The results of KMnO_4 modification on oligonucleotide I performed in 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA (TE) buffer are shown in Figure 2 (Example 1A). Thymines in the middle segment (T_{12} - T_{17}) are less reactive toward KMnO_4 than thymines on either side (Figure 2, lane 2), consistent with the middle segment being base-paired to the 5' segment, making a Watson-Crick duplex. With increasing concentration of Mg^{++} ions, thymines in the 3' segment (T_{23} - T_{28} ; bracket) become more and more resistant to KMnO_4 modification and the protection of thymines in the middle segment becomes more pronounced (Figure 2,

lanes 3-5). In 20 mM $MgCl_2$ (Figure 2, lane 5), the overall reactivity pattern is consistent with an intramolecular triplex having TAT base triplets, as expected, with only the thymines that occupy the putative loop regions (T_9-T_{11} and $T_{18}-T_{22}$) being sensitive to $KMnO_4$ modification and thus providing an internal control for the modification. The first two thymines at the 5' end remain hyperreactive toward $KMnO_4$ throughout all conditions, indicating that these bases are unpaired possibly because of "fraying," i.e., breathing or transient un-pairing of normally paired bases at an end of a molecule.

In contrast to oligonucleotide I, oligonucleotide II has the potential to form an intramolecular triplex with AAT base triplets. The reactivity patterns of oligonucleotide II in 50 mM sodium cacodylate (pH 7.1) buffer (Figure 3, Example 1B) support a triplex conformation. In addition to the thymines occupying the two potential loops (T_9-T_{12} and $T_{21}-T_{24}$), the first four thymines (T_1-T_4) at the 5' end are also reactive toward $KMnO_4$ (Figure 3, lanes 2-4, arrows), suggesting that these bases are at least transiently unpaired or frayed. The lesser reactivity of internal thymines in the 5' segment (T_5-T_8) suggests the formation of a Watson-Crick duplex within this region. The reactivity of oligonucleotide II toward DEPC in the same buffer is shown in lanes 6-8 (Figure 3). All adenines of the 3'-segment are equally reactive, whereas those on the middle segment are not; $A_{13}-A_{16}$ are protected from DEPC modification, and $A_{17}-A_{20}$ are reactive. The DEPC protection of $A_{13}-A_{16}$ residues can be attributed to the formation of AAT base triplets. The reactivity pattern of these adenines does not change with the addition of Mg^{++} ions, indicating that the formation of AAT triplets does not require Mg^{++} ions. This finding is analogous to the behavior of GGC base

triplets, detection of which does not require Mg^{++} ions in the medium (Chen, 1991). The reactivity of $A_{17}A_{20}$ indicates that these adenines are not stably involved in the formation of A:AT triplets, apparently because of fraying of the homologous Watson-Crick duplex in this region as deduced from $KMnO_4$ reactivity.

Attempts to construct intermolecular, as opposed to intramolecular, duplex or triplex pairing schemes for either oligonucleotides I or II that were consistent with the observed reactivities were unsuccessful. For example, an intermolecular duplex structure for oligonucleotide I should result in protection of thymines next to the adenine tract (T_9-T_{16}), not hyperreactivity as is observed.

Oligonucleotide III has two adjacent potential triple-helix forming motifs, one making T:AT and the other making A:AT triplets. Thymines T_4-T_7 on the 5' segment are unreactive toward $KMnO_4$ in 50 mM sodium cacodylate (pH 7.1) buffer (Example 1C; Figure 4A, lane 3), suggesting that they are in a Watson-Crick duplex. In contrast to T_4-T_7 , $T_{24}-T_{26}$ on the 3' segment are modified by $KMnO_4$ (Figure 4A, lane 3), indicative of single-stranded character. With the addition of Mg^{++} ions to the medium, the reactivity of these reactive thymines ($T_{24}-T_{26}$) is reduced (Figure 4A, lanes 4-6, bracket), suggesting triplex formation with T:AT base triplets. In addition to $T_{24}-T_{26}$, thymine T_{21} shows Mg^{++} -dependent resistance toward modification. Because it is flanked by two guanines in the loop, the protection is probably due to stacking with adjacent guanines induced by Mg^{++} -dependent folding. Such protection from $KMnO_4$ of thymines primarily residing in loops has been observed previously (Haner & Dervan, 1990).

Oligonucleotide III was modified with DEPC in the same buffer to detect whether A:AT triplets are

formed, in addition to TAT triplets; the results are shown in lanes 9-12 of Figure 4A (Example 1C). Adenines 13-15 are protected from DEPC (open arrowheads), suggesting the formation of AAT base triplets. As observed for oligonucleotide II, the reactivity of these less-reactive adenines (A_{13} - A_{15}) is not sensitive to the presence of Mg^{++} ions. Because it is located in the loop region, the enhanced reactivity of A_{11} (bottom closed arrowhead) is expected, and it serves as an internal control for the modification. However, the high reactivity of A_{12} (a base at the extreme end of the helix) is unexpected and could be due to the distortion caused by the loop making it unavailable for base pairing. Adenines at the 3' end (A_{27} - A_{30}) are reactive whereas adenines at the 5' end ($A_{1,3}$) are less reactive, as clearly seen in Figure 4B (bottom of the gel, lanes 1 and 2), where DEPC modification was performed at different temperatures. In the absence of Mg^{++} ions the less reactive adenines (A_{13} - A_{15} ; bracket) become reactive at approximately 37°C (Figure 4B, lane 3), suggesting the melting of AAT base triplets. The addition of Mg^{++} ions raises the melting temperature, so that maximal reactivity is seen at 55°C instead of 37°C (Figure 4B, lane 9). Thus, the results indicate that Mg^{++} ions are not essential for the formation of AAT base triplets, but their stability is enhanced by Mg^{++} ions. Taken together, the results of $KMnO_4$ and DEPC modifications suggest the presence of both PyPuPy (TAT) and PuPuPy (AAT) base triplets within oligonucleotide III in the presence of Mg^{++} ions.

Like oligonucleotide III, oligonucleotide IV also has the potential to form a triplex having juxtaposed PuPuPy and PyPuPy motifs, but with different base triplets (TAT and GGC). The results of $KMnO_4$ modification of oligonucleotide IV are shown

in Figure 5A (Example 1D). Thymines on the 3' segment (T_{25} - T_{28}) are reactive toward $KMnO_4$ in TE buffer (Figure 5A, lane 3, bracket), indicating that they are in a single-stranded form. However, with the addition of Mg^{++} ions, T_{25} - T_{28} become less reactive (Figure 5A, lanes 4-6), suggesting the formation of TAT base triplets. On the other hand, at all Mg^{++} ion concentrations, T_{17} - T_{20} remain less reactive, consistent with their involvement in a duplex. The reactivities of T_9 - T_{12} and T_{22} - T_{23} do not change under any condition, indicating their singlestranded nature in the folded (Figure 5A, lane 6) as well as unfolded structure (Figure 5A, lane 3).

The results of DMS modification of oligonucleotide IV performed in TE buffer at different temperatures are shown in Figure 5B (Example 1D). In the absence of Mg^{++} ions, G_{13} - G_{16} become more reactive at temperatures above 23°C (Figure 5B, lanes 1-6), suggesting the presence of GGC base triplets at 23°C or below. In the presence of Mg^{++} ions, these guanines are less reactive at 23°C than they are in the absence of Mg^{++} , indicating that, analogous to AAT base triplets, the stability of GGC base triplets is also enhanced in the presence of Mg^{++} even though Mg^{++} is not essential for their formation.

Thus the results of $KMnO_4$ and DMS modifications indicate the formation of a triple-helix containing both TAT and GGC base triplets in oligonucleotide IV. Comparison of the melting temperatures for oligonucleotides III and IV suggests that AAT base triplets are more stable than GGC base triplets.

The potential triple helix-forming regions of oligonucleotide V consist of mixed purines and mixed pyrimidines. Unlike the case for other oligonucleotides, the formation of an intramolecular triplex by oligonucleotide V is expected to be pH-dependent because of the involvement of C^+ GC base triplets.

The results of KMnO_4 modification of oligonucleotide V at different pH values are shown in Figure 6A (Example 1E). At pH 4.5, both T_{26} and T_{28} on the 3' segment are protected from KMnO_4 modification (Figure 6A, lane 3, arrowheads) whereas at pH 6 and 7 these two thymines are modified. This pH-dependent reactivity is consistent with the formation of TAT base triplets in the 3' segment at a pH permitting C⁺GC triplets to form. In 10 mM Mg^{++} , T_{26} and T_{28} remain unreactive at both pH 6 and 7.

The formation of AAT and GGC base triplets in oligonucleotide V is detected by reacting with DEPC and DMS, respectively (Example 1E). As seen in lanes 3-7 of Figure 6B, A_{14} and A_{16} are less reactive toward DEPC (open arrowheads) than A_{24} (closed arrowhead), consistent with A_{14} and A_{16} being involved in AAT base triplets and A_{24} residing in a loop. Both A_2 and A_4 are less reactive, consistent with their involvement in TAT base triplets. With DMS modification (Figure 6B, lanes 8-12), G_{13} and G_{15} remain less reactive than the internal control G, (in a loop; closed arrowhead), suggesting the involvement of G_{13} and G_{15} in GGC base triplets. Here again, the formation of AAT and GGC base triplets is not dependent on Mg^{++} ions. Furthermore, as expected, their formation is independent of pH as shown by the identical reactivity patterns observed for both DEPC and DMS modifications at pH 4.5 and 7.0 (Figure 6B, compare lanes 4 and 5 with 6 and 7, and lanes 9 and 10 with 11 and 12).

Figure 6C (Example 1E) shows the melting of oligonucleotide V as monitored by DEPC reactivity. As was the case with Pu•PuPy base triplets in oligonucleotides III and IV, A•AT triplets (at A_{14} and A_{16}) melt at a higher temperature in the presence of Mg^{++} ions ($>70^\circ\text{C}$ at 10 mM Mg^{++} compared to $<55^\circ\text{C}$ without Mg^{++} ; Figure 6C, compare lanes 3-6 with lanes 7-10

(arrow heads)). On the other hand, G₃ and A₄ show a transition to increased reactivity well below 70°C even in the presence of Mg⁺⁺ ions, indicating the melting of C⁺-GC and T-AT base triplets. Therefore, in the presence of Mg⁺⁺ ions, A-AT base triplets in this mixed purine tract appear to be more stable than T-AT base triplets. The different melting temperatures observed for A-AT base triplets generated within oligonucleotides V and III could be due to a sequence effect (oligonucleotide V contains 50% GC). In summary, the results of chemical modifications of oligonucleotide V show the formation of intramolecular triplexes utilizing alternate strands of duplex DNA containing all four bases.

Experiments performed in support of the present invention show that using two types of base triplets, PuPuPy and PyPuPy, a single strand consisting of oligopurine- and oligopyrimidine-blocks bind simultaneously to a duplex DNA, where the duplex contains adjacent tracts of oligopurines and oligopyrimidines. This binding is via both PuPuPy and PyPuPy base triplets, as exemplified in Figure 1a(iii).

In Figure 1a(iii), the oligopyrimidine block of the single strand is parallel to one purine tract of the duplex DNA and its oligopurine block is anti-parallel to the other purine tract on the alternate strand of the duplex. Unlike previous methods, where polarity considerations required the design of an oligonucleotide with an unnatural 3'-3' linkage (Horne & Dervan, 1990), the present invention requires no special junction for this polarity of binding between the oligopurine and oligopyrimidine blocks of the single strand occupying the major groove of both oligopurine and oligopyrimidine tracts.

The above experiments support that triple-helix formation can occur as described above, at tandem oligopurine and oligopyrimidine tracts. The reactivity patterns of the above-discussed oligonucleotides, as well as the deduced structures for each, are summarized in Figure 7.

Two different hydrogen bonding schemes have been proposed for the AAT base triplet (Beal & Dervan, 1991), one with two hydrogen bonds involving N-7 and N-6 of the Watson-Crick adenine, and the other with only one hydrogen bond forming at the N-6 of the same adenine. Because the Watson-Crick adenine is protected from DEPC, which reacts primarily at the N-7 of purines, the experiments performed in support of the present invention suggest that the AAT base triplet has two hydrogen bonds involving both N-7 and N-6 of the Watson-Crick adenine.

Oligonucleotides III-V, containing tandem tracts of oligopurines and oligopyrimidines in different base combinations, exhibit chemical reactivity patterns that are in agreement with an intramolecular triplex structure forming both PuPuPy and PyPuPy base triplets simultaneously. In each case, the reactivity patterns described above are consistent with the bases of a single-stranded region occupying the major groove of the duplex hairpin, base-pairing to the purine tract in each Watson-Crick strand, as depicted in Figure 1 (A and B).

The results of chemical modification performed at different temperatures suggest that Mg^{++} ions enhance the stability of PuPuPy base triplets, although their formation does not require Mg^{++} .

The above experiments suggest that both PyPuPy and PuPuPy base triplets can be incorporated into the triplex by using a third strand consisting of oligopurine and oligopyrimidine blocks. This

approach allows triplex formation at sequences consisting of tandem tracts of oligopurines and oligopyrimidines. Designing oligonucleotides to bind purine tracts of alternate strands appears to be a viable strategy for triplex formation involving any sequence of DNA, relaxing the restriction that target sequences be long (>10 nucleotides) oligopurine tracts.

10 II. Diagnostic and Therapeutic Application of the Oligonucleotides of the Present Invention.

 A. Band-Shift Assays.

 The third strand oligonucleotides of the present invention can be used in a number of diagnostic and therapeutic applications. One diagnostic application of the oligonucleotides of the present invention is to provide a means of site-directed cleavage at a target duplex by coupling oligonucleotides to cleaving agents. One therapeutic application of the present invention is targeting a selected gene for inactivation, e.g., by suppression of mRNA synthesis from the target gene (Cooney et al., 1989; Orson et al., 1991; Postel et al., 1991). In both of the above applications a defined oligonucleotide is directed at the duplex DNA target to form a triple helix.

 The triple-helix approach for targeting DNA has had limited use to date because of the requirement for long homopurine target sequences. Prior to the present invention, triplex formation at an oligopurine•oligopyrimidine typically required a single strand consisting of only either pyrimidines or only purines; the ability to use alternating tracts of oligopurine and oligopyrimidine sequences, as described above, obviates this requirement.

 Oligonucleotides of the present invention are tested for triplex formation with duplex target

sequences using band shift assays (Example 2). Typically, high percentage polyacrylamide gels are used for band-shift analysis where denaturing conditions (Ausubel et al.; Sauer et al.; Sambrook et al.) are adjusted to reduce any background of non-specific binding.

In general a target duplex oligonucleotide is used initially for band shift analysis. The duplex target is radiolabeled and mixed with a third strand oligonucleotide which is being tested for its ability to form triplex structures with the target duplex. Oligonucleotides are evaluated for their ability to shift the mobility of the duplex oligonucleotide.

Triplex formation is indicated in the band shift assay by a decreased mobility in the gel of the labeled triplex structure relative to the labeled duplex structure, i.e., triplexes appear as bands having apparent higher molecular weight.

A number of controls are performed to assure the oligonucleotide specificity of triplex-formation. First, pre-mixing the third strand oligonucleotide with a complementary DNA strand, prior to triplex formation, should block the ability of oligonucleotide to form a triplex with the radiolabeled duplex. The complementary strand added to the duplex alone should neither have an effect on the mobility of the radiolabeled duplex DNA, nor be able to block as complementary oligonucleotides when added to reaction mixtures after triplex formation has occurred. Second, oligonucleotides having similar purine and pyrimidine compositions to the third strand oligonucleotide, but which have a random mix of the purine and pyrimidines instead of to the sequence specified by the duplex target are prepared. The random sequence oligonucleotides should neither affect the mobility of labeled duplex

target nor interfere with the band-shift caused by sequence-specific third-strand oligonucleotide.

Similar experiments can be employed in which the single strand oligonucleotides are radiolabeled and the duplex added to them. The same logic is used in establishing control experiments as outlined for the radiolabeled duplex DNA.

10 B. Cleaving Agents Covalently Attached to Third Strand Oligonucleotides.

Typically, inhibition of gene expression via triplex formation has been attempted only with oligonucleotides carrying no chemical cleaving groups (Cooney et al., 1989; Orson et al., 1991; Postel et al., 1991). This is also true for antisense inhibition of gene expression (Matsukura et al., 1989; Agrawal et al., 1989; Zamichnik et al., 1986; Rittner and Sczakiel, 1991). To be effective, antisense agents of this type must continuously bind to all the target molecules in such a way as to inactivate them. However, the oligonucleotides of the present invention can be equipped with chemical cleaving groups.

The advantage of using a chemical cleaving moiety is that, in addition to binding, the reagent permanently inactivates the target nucleic acid sequences. This method is applicable to any double strand DNA target, and can be also be applied to selected target RNA molecules (see below). The present invention is particularly useful in targeting viral genomes (free replicating or integrated) for inactivation.

35 An example of a family of such viruses is the family of human immunodeficiency viruses (HIV), in particular, HIV-1, which appears to be the etiological agent responsible for AIDS.

Potential third strand oligonucleotides are tested for triplex formation as described above.

The sequences of the oligonucleotides are chosen based on target sequences having tracts of oligo-purines oligopyrimidines. For example, Figure 9A-9C illustrate three potential sites for targeting within the LTR region of HIV-1 with single-stranded oligonucleotides. Figure 10 shows the sequences of oligonucleotides A 1 (SEQ ID NO:9) and 2 (SEQ ID NO:10), B 1 (SEQ ID NO:11) and 2 (SEQ ID NO:12), and C 1 (SEQ ID NO:13) and 2 (SEQ ID NO:14) designed to target sequences of Figure 9A, 9B, and 9C, respectively. Next to each of these oligonucleotides (Figure 10) is the general pattern of base triplets expected to form when triplexes are formed. Oligonucleotides K (SEQ ID NO:15), L (SEQ ID NO:16), and M (SEQ ID NO:17) are the control oligonucleotides (Figure 10).

All three target sites are located in the control region of the LTR, i.e., upstream of the transcription initiation site and therefore do not interact with mRNA sequences to function as anti-sense mediators. The potential target sites A, B, and C (Figure 9) have different triplex forming motifs: Site A, consisting exclusively of purines is targeted with oligonucleotides A-1 and A-2 (Figure 10), which are capable of forming triplexes with Pu•PuPy and Py•PuPy base triplets, respectively. Site B consists of a tract of pyrimidine residues flanked by two purine tracts and is targeted with oligonucleotides B-1 and B-2 (Figure 10), which have the correct polarities for triplex formation. Site C has some pyrimidines buried in a highly purine-rich sequence, and oligonucleotides C-1 and C-2 (Figure 10) is directed toward site C. Oligonucleotides K, L, and M, each having incorrect polarity for triplex formation at sites A, B, and C, respectively, are used as controls. Test oligomers

with and without phenanthroline are used to assess the effect of cleavage.

A number of cleaving agents may be used in the present invention including the following chemical moieties which are capable of cleaving nucleic acid substrates: phenanthroline (Chen et al., 1986, 1987; François et al., 1989; Ebright et al., 1990), Fe(II)-EDTA (Dreyer et al., 1985; Dervan, 1986; Moser et al., 1987; Maher et al., 1989; Sluka et al., 1987), Cu(II)-bipyridine, Cu(II)-terpyridine, and Zn(II) (Modak et al., 1991; Eichhorn et al., 1971; ; Breslow et al., 1989). These chemical cleaving moieties can be employed in the present invention as exemplified below with reference to the phenanthroline moiety.

The cleaving agent 1,10-phenanthroline is attached to the third strand oligonucleotides as described in Example 3.

The ability of test oligonucleotides to form triplexes and carry out cleavage at their designated target sites is assayed by determining the site-specific cleavage induced at the target sequences by test oligonucleotides equipped with the phenanthroline moiety (Example 4). For the oligonucleotides shown in Figure 10, pHIV-1CAT is linearized with, end-labeled, and subjected to a second restriction digest to obtain a uniquely labeled DNA fragment. After gel purification, this DNA fragment is mixed with a phenanthroline modified oligonucleotide. Cleavage products are resolved on sequencing gels along with the products of sequencing reactions. This method maps the site of triplex formation, and provides an indication of the cleavage efficiency (detected by counting the radioactivity of excised gel bands); the method allows the quantitation of the efficiency of triplex formation.

In vitro triplex formation can also be tested using the band shift assay described above, where cleavage of the duplex target is evidenced by increased mobility of the fragments of the labeled
5 duplexes in the gel. For these assays polyacrylamide gels run under DNA-denaturing conditions (Ausubel et al.; Sambrook et al.) can be used.

To examine the ability of the phenanthroline-coupled-oligonucleotides to inhibit gene expression
10 by *in vivo* triplex formation, transient expression of the CAT gene under the direction of HIV-1 LTR in HeLa cells is used. HeLa cells are transfected with PHIV-1 CAT, using the DEAE-dextran technique (Queen and Baltimore, 1983) or $\text{Ca}_3(\text{PO}_4)_2$ technique. Oligo-
15 nucleotides carrying phenanthroline are complexed with CuSO_4 before they are introduced to the cell medium. Approximately twelve hours after transfection, the cells are incubated with a phenanthroline-coupled-oligonucleotide, as described by Postel et
20 al. (1991). Mercaptopropionic acid or ascorbic acid are then supplied to the medium approximately 12 hr after the oligonucleotide treatment.

Mitomycin C is added to the medium to induce CAT expression; since the HIV-1 LTR is under the
25 influence of NF- κ B, the expression of CAT activity can be induced by treating with either ultraviolet light or mitomycin C. Cells are harvested at 12 and 24 hr after mitomycin C addition, and CAT activities determined as described by Gorman et al. (1982).
30 CAT expression is compared to controls including cells that have been exposed to control phenanthroline-coupled-oligonucleotides (K, L, and M), phenanthroline-coupled-oligonucleotides (SEQ ID NO:9-SEQ ID NO:17) without complexing to CuSO_4 , and cells
35 without oligonucleotide treatment. Successful cleavage of the target by the duplex-targeted third strand oligonucleotide reduces levels of CAT expres-

sion relative to controls, in particular, relative to phenanthroline-coupled-oligonucleotides (K, L, and M) complexed to CuSO_4 .

5 Other HIV-reporter gene constructs can be used in the above experiments (e.g., Cross et al.), including HIV-LTR constructs containing a luciferase (de Wet et al.) reporter gene.

C. Oligonucleotide Inhibition.

10 In addition to the above-described site directed cleavage of target DNA duplexes by the oligonucleotides of the present invention, oligonucleotides can be constructed to target duplex DNA regions in order to block expression -- without cleavage.

15 Target sites can be chosen in the control region of the genes, e.g., in the transcription initiation site or binding regions of regulatory proteins (Helene and Toulme, 1990; Birg et al., 1990; Postel et al., 1991; Cooney et al., 1988).
20 These oligonucleotides can also interact with mRNA sequences to function as antisense mediators (see below).

Single-stranded DNA can also be used as a target nucleic acid for oligonucleotides of the present invention. For example, two oligonucleo-
25 tides can be used for target-directed binding. Alternately, oligonucleotides similar to those described below (TDAs) can be used for the binding or binding/cleavage of target sequences contained in
30 single-stranded DNA molecules.

D. Triplex-Directed Antisense Oligonucleotides.

35 Single-stranded oligonucleotides of the present invention can also be used in an anti-sense expression inhibition technique, where the single-stranded oligonucleotides are capable of forming triplex structures with target mRNAs (Giovannangeli et al.,

1991). This triplex-directed antisense (TDA) approach may be more effective than a duplex (conventional antisense approach) in arresting biological processes such as translation and reverse transcription. Triplex formation in this fashion is highly selective and of high affinity and may not be a substrate for enzymes such as helicases. The action of such helicases can be a potential problem in the conventional antisense approach.

10 Experiments performed in support of the present invention, see intramolecular triplexes described above, indicate the feasibility of triple helix formation between a single-stranded RNA and a singlestranded DNA which contains complementary
15 regions capable of forming a duplex. The complexation of single-stranded oligonucleotides with circular oligonucleotides has been studied (Kool, 1991) and complexation by circular oligonucleotides that can form triplexes with the single strands has
20 been found to be more selective and stable than by the linear complementary oligonucleotides.

The TDA approach of the present invention is applicable to any target mRNA molecule; an example of targeting HIV mRNA is presented below.

25 Three TDA target sites (D, E, and F) within the 5786-8887 nucleotide region of the HIV-1 genome are presented in Figure 12A. The figure also shows a target region for each mRNA sequence (underlined). TDA oligonucleotides directed to these sequences are
30 presented as oligonucleotides D1, D2, E1, E2, and F1, F2 in Figure 12B. Oligonucleotides complementary to the target regions, i.e., oligonucleotides designed to function as conventional antisense agents, are presented as oligonucleotides D_x, E_x, and
35 F_x in Figure 12B.

For each of these target mRNA sequences (Figure 12A, underlined), oligonucleotides capable of

forming triplexes are synthesized. Two different triplexes, one with Pu•PuPy and the other with Py•PuPy base triplets, are possible for each target (Figure 12B). To avoid nonspecific interactions with loop residues of hairpins, connecting sequences ("loops") are synthesized with the "UNI-LINK AMINO-MODIFIER" (Clonetech, Palo Alto CA), which lacks a base and a sugar attached to the phosphate. Because of the continuity and the proper interphosphate spacing of the backbone rendered by "UNI-LINK AMINOMODIFIER" triplex formation is not be affected. In Figure 12B, "X" indicates an abasic "UNI-LINK AMINOMODIFIER" site.

The *in vivo* effect of TDA oligonucleotides are assayed by detecting the expression of gp120 (envelope glycoprotein) of HIV. HeLa T4+ cells (AIDS Research and Reference Program) are transfected with the pDOLHIVenv (available from the AIDS Research and Reference Program) plasmid and selected for β -kanamycin resistance. The pDOLHIVenv plasmid has open reading frames for Env, Tat, and Rev proteins (nucleotides 5786-8887 of the HIV-1 genome). β -Kanamycin-resistant cells are picked and expanded. These cells are then incubated with a selected TDA oligonucleotide for 24 hr. The level of expression of envelope proteins is assayed by a syncytium (giant cell) formation assay carried out in microtiter plates (Putney, et al., 1989). Because syncytium induction by HIV is mediated by the interaction of the CD4 molecule with gp120 on the surface of cells, the number of syncytia is directly proportional to the amount of expressed gp120.

To compare the effectiveness of the TDA oligonucleotides and conventional antisense oligonucleotides, oligonucleotides D2, E2, and F2, which are capable of direct binding to mRNA, are used as controls in gp120 expression experiments.

Alternatively, an indirect immunofluorescence assay is employed to detect the level of gp120 expression. In this case, an antiserum to gp120_{RF} (available from the AIDS Research and Reference Program) is used as the primary antibody in a standard indirect immunofluorescence antigen detection assay (Harlow et al.). Rhodamine isothiocyanate-conjugated rabbit anti-goat immunoglobulin G (Pierce, Rockford, IL) is used as the secondary detection antibody.

TDA oligonucleotides can be targeted to regulatory regions, as described above, or to other regions, such as splice sites in pre-mRNA molecules.

15 E. Relative Propensities of Formation of Different Base Triplets.

In addition to A•AT and G•GC, several unusual base triplets have been documented. These include A+•GC, base triplets in DNA and I•AU and I•GC in RNA. Triple helix formation is extremely sequence-specific and bases that can form hydrogen bonds with Watson-Crick base pairs can form triplexes. However, the relative stabilities which may vary from one base triplex to the other is important in designing single strand probes for triplex formation. The affinity cleaving assay described above is used to detect the relative propensity of triplex formation involving oligonucleotides containing the above unusual base triplets.

30 For example, the propensity of triplex formation by I•GC with respect to that of G•GC is assayed by comparing the cleavage efficiency induced by oligonucleotide I1 and G1 (Figure 15B). In these two cases the polarity of the third strand as well as the type of neighboring base triplets would be the same except for variants, i.e., I•GC and G•GC base triplets. Thus the difference of cleavage efficiency induced by these two oligonucleotides simply

reflect the propensity of triplex formation by I•GC and G•GC base triplets. Similarly, the propensity of the formation of I•AT triplet with respect to that of A•AT is be assayed using oligonucleotide I2 and A1 (Figure 15B).

Because I•GC and I•AT triplets have been documented, oligonucleotide I1 will be used to detect targeting in the duplex sequences shown in Figure 15A.

10

F. General Considerations Concerning the Oligonucleotides of the Present Invention.

The oligonucleotides of the present invention comprise tandem, alternating tracts of purine and pyrimidine sequences, where the length of each tract may vary but contains at least one purine or pyrimidine base (Example 4B). Typically, the tracts of purines and pyrimidines contain at least 4 purine bases followed by at least 4 pyrimidine bases. The number of bases in the tracts of purines and pyrimidines do not have to be equal, for example, a tract of 4 purines may be attached to a tract of 9 pyrimidines, or the oligonucleotide may comprise tandem tracts of 4 purines, 9 pyrimidines, 2 purines, 5 pyrimidines. The typical length of targets for the oligonucleotides of the present invention are from about 8 nucleotides to about 60 nucleotides; the length of the target is used, in part, to determine target specificity and triplex stability.

Cellular uptake of the ³²P-labeled oligonucleotides is evaluated essentially as described by Postel et al. Stability of the oligonucleotides in vivo can be evaluated by freeze-drying aliquots from the medium and from the nuclear and cytoplasmic fractions and analyzing the resuspended aliquots on 12% sequencing gels (Postel et al.).

The delivery of any of the oligonucleotides of the present invention into cells can be facilitated

using established techniques such as scrape loading, electroporation, and microinjection. Alternatively, oligonucleotides can be encapsulated within liposomes and these loaded liposomes incubated with
5 cells.

The ends of oligonucleotides can be protected by attaching intercalators; this has been shown to make oligonucleotides resistant to exonucleases and facilitates cell permeation (Cazenave et al., 1987).
10 The linking of intercalators improves the exonuclease resistance of oligonucleotides and further provides additional binding energy (because of intercalation) without perturbing the specificity of recognition of complementary sequences. Backbone
15 modifications such as methylphosphonates and phosphothioates not only make oligonucleotides endonuclease resistant, but the neutral backbones also facilitate cell permeation (Matteucci et al. (1991); Miller et al.).

20 Oligonucleotides at the 5' and 3' ends can be derivatized with two different linking molecules using different chemistry. Such derivatization is performed by using "3'-AMINE-ON CPG" (Clontech) solid supports to incorporate a functional amino
25 group at the 3' end, and incorporating a thiol group at the 5' end as described above. Because deprotection procedures for the two functional groups are different and independent from each other, derivatization at the two ends is performed at two stages of
30 oligonucleotide synthesis.

TDA oligonucleotides can be derivatized at one or both ends to modify the oligonucleotides to provide a cleavage function, as described above; RNA targets can be cleaved using the cleaving
35 moieties described above, including 1,10 phenanthroline.

For cleavage reactions carried out in cells in culture, the intracellular reduction potential can be modulated using N-acetyl cysteine, which increases the intracellular glutathione level (Roederer et al., 1990; Kalebic et al., 1991). Such manipulation of the intracellular reduction should assist in keeping, for example, a copper atom of a cleaving agent in the reduced state.

The level of expression of cytoplasmic mRNA corresponding to target sequences can be evaluated by standard methods including: Northern analysis using a target mRNA specific probe (Ausubel et al.; Sambrook et al.; Postel et al.); dot-blot hybridizations using target mRNA specific probes (Ausubel et al.); and polymerase chain reaction (Mullis; Mullis et al.) using target mRNA specific probes.

For increasing binding affinity of oligonucleotides for their targets, substitution of Br5U (5-bromo-uracil) for thymines in the third strand increases binding affinity when a triplex is formed. Therefore, thymines that form Hoogsteen hydrogen bonds can be replaced with Br5U during chemical synthesis. Further, the substitution of Me5C (5-methyl-cytosine) in place of cytosines in the third strand is useful for effective targeting at physiological pH.

III. Utility.

The ability of the oligonucleotides of the present invention to recognize targets having combinations of tracts of purines and pyrimidines provides flexibility for techniques based on triplex formation, including third strand inactivation of a target duplex gene and anti-sense inactivation (Helene and Toulme, 1990).

Further, the cleaving reagents of the present invention provide means for a method of cleaving RNA

targets at specific sites. Such cleavage is useful for the analysis of RNA structure and function as well as diagnostic analyses. One example of a diagnostic application is to isolate RNA from a cell
5 infected with a particular RNA virus. Total or poly-A+ RNA (Ausubel et al.) is end labeled. The RNA is then isolated away from free label and the amount of incorporated label estimated, for example, by scintillation counting. The labeled RNA is then
10 treated with an RNA cleaving agent, such as a TDA oligonucleotide combined with a chemical cleaving moiety, and the amount of liberated label is used as an indicator of the concentration of RNA contain the RNA-binding protein cognate binding site. The
15 cleaving reagents of the present invention are particularly desirable for use with DNA virus targets and RNA virus targets or their pro-viral DNA forms: for example, cleaving HIV genomic RNA or pro-viral DNA.

20 Oligonucleotides of the present invention, capable of forming triple-helix structures with single-strand or duplex DNA, can also be labeled and used as probes, or, when carrying cleaving agents, can be used as site-specific cleaving agents.

25 The cleaving reagents of the present invention are also useful in a method of inhibiting expression of RNA viral (e.g., HIV) antigens in cells infected with the virus. In this application, infected cells are exposed to an oligonucleotide (DNA duplex
30 directed, antisense, or TDA) modified to contain a cleaving moiety (i.e., the reagent), at a reagent concentration effective to produce reduction in viral antigen expression in the infected cells (Wang et al., 1988, 1989; Crowe et al., 1990). Examples
35 of such reagents for anti-HIV agents have been described above.

A combined use of RNA cleaving (or inhibiting) oligonucleotides combined with the above-described DNA duplex directed cleaving (or inhibiting) oligonucleotides may provide a two-pronged therapeutic attack against viral diseases by providing cleavage of viral RNA and DNA or pro-viral DNA genomes. A major advantage of targeting the DNA pro-virus associated with an RNA virus is that typically only one, or a few copies, of integrated, transcriptionally active DNA are present per cell in contrast to many copies of mRNA which may be present in an infected cell (Soma et al., 1988).

The following examples illustrate, but in no way are intended to limit the present invention.

Materials and Methods

Oligonucleotides were synthesized on an Applied Biosystems Model 381 B automated DNA synthesizer (Applied Biosystems, Foster City CA) using cyanoethyl phosphoramidites. After deprotection, they were purified by electrophoresis on denaturing 20% polyacrylamide gels (Ausubel et al.; Sambrook et al.).

Purified oligonucleotides were labeled at 5' ends using T₄ polynucleotide kinase (United States Biochemicals, Cleveland OH) and ³²P-γ-ATP (Du Pont-NEN, Boston MA) according to the method of Maxam & Gilbert (1980). Unincorporated ATP was separated (Ausubel et al.; Sambrook et al.) from labeled oligonucleotides by passing the labeling reaction mixture through two successive "SEPHADEX G-50" spin columns (Pharmacia, Piscataway NJ) equilibrated in 10 mM Tris-HCl and 0.1 mM EDTA (pH 7.5).

Modification of oligonucleotides using potassium permanganate (KMnO₄) was performed essentially as

described by Rubin & Schmid (1980). A radiolabeled oligonucleotide (approximately 100 ng) was equilibrated in 9 ml. of the reaction buffer (see below) at 4°C for 10 min, then 1 ml. of 5 mM KMnO_4 (freshly diluted from a 100 mM stock stored at 4°C) was added. After 10 min of incubation at 4°C, the reaction was stopped by adding 2 ml. of neat allyl alcohol.

Modification of oligonucleotides using diethyl pyrocarbonate (DEPC) was performed essentially as described by Johnston & Rich (1985) or Herr (1985). An end-labeled oligonucleotide (approximately 100 ng) was equilibrated in 97.5 ml. of a reaction buffer (see below) for 10 min at 4°C. Then 2.5 ml. of DEPC (Aldrich, Milwaukee WI) was added, vortexed briefly, and the incubation was continued for 30 min.

Modification of oligonucleotides using dimethyl sulfate (DMS) was performed essentially as described by Maxam & Gilbert (1980). One microliter of a 1:200 dilution of DMS in water (freshly prepared) was added to an end-labeled oligonucleotide (approximately 100 ng) equilibrated in 9 ml. of reaction buffer (see below) at 4°C, and the modification was carried out for 10 min at the same temperature.

For modifications performed at higher temperatures than noted above, the reaction times were decreased accordingly in order to control excess modification.

The modification reactions were stopped by two ethanol precipitations except in the case of KMnO_4 , as noted above.

DNA pellets were thoroughly washed with 70% ethanol, dried, and treated with hot piperidine (1 M, 90°C for 30 min). KMnO_4 -modified DNA was treated with piperidine directly, without ethanol precipitation. After removing piperidine by vacuum evapora-

tion, DNAs were resuspended in formamide loading buffer, and the cleavage products were resolved on 20% sequencing gels. Sequencing reactions on oligonucleotides were performed according to Williamson & Celander (1990).

Example 1

Intramolecular Triplex Formation

A. KMnO_4 reactivity of oligonucleotide I.

10 KMnO_4 modification was performed in 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA (TE) buffer at 4°C in the absence (Figure 2, lane 2) and presence of MgCl_2 (Figure 2, lanes 3-5, concentrations as indicated). Figure 2, lane 1 is the A+G sequencing
15 reaction of oligonucleotide I (Maxam and Gilbert). Modification of thymines within the bracket, "{" indicated in lane 1, is sensitive to MgCl_2 . In Figure 2, the open and closed bars, indicated on the right, represent WatsonCrick duplex region; single
20 lines show single-stranded regions; and the hatched bar indicates the third "strand" of the triplex.

B. KMnO_4 and DEPC reactivity of oligonucleotide II.

25 Both modifications were performed at 4°C in 50 mM sodium cacodylate buffer (pH 7.1) with or without MgCl_2 . In Figure 3, lanes 1 and 5 are the A+G sequencing reaction for oligonucleotide II; lanes 2-4, KMnO_4 modification; and, lanes 6-8, DEPC modification. MgCl_2 concentrations were as shown in the
30 figure. Closed arrows show hyperreactive thymines (T_2 - T_4) at the 5' end of the oligonucleotide. Open arrowheads show adenines (A_{13} - A_{16}) that are protected from DEPC modification, whereas closed arrowheads represent reactive adenines (A_{17} - A_{20}). Helical and
35 loop regions are designated as described above for Figure 2.

C. KMnO_4 and DEPC reactivity of oligonucleotide III.

1. KMnO_4 and DEPC Modifications.

5 KMnO_4 (Figure 4A, lanes 3-6) and DEPC (Figure 4A, lanes 9-12) modifications performed in 50 mM sodium cacodylate buffer (pH 7.1) at 4°C. The bracket (]) indicates thymines whose reactivity pattern is sensitive to MgCl_2 . In Figure 4A, lanes 1 and 7 are the G sequencing reaction, and lanes 2 and 8 are the A+G sequencing reaction. MgCl_2 concentrations were as indicated in the figure. Closed arrowheads indicate adenines that are reactive toward DEPC, whereas those indicated by open arrowheads are protected.

2. DEPC reactivity as a function of temperature.

DEPC reactivity as a function of temperature was examined in the absence (Figure 4B, lanes 1-6) and presence (Figure 4C, lanes 7-10) of 10 mM $MgCl_2$. Temperatures were as indicated at the top of the figure. Helical and loop regions are as described above for Figure 2.

10 D. $KMnO_4$ and DMS reactivity of oligonucleotide IV.

1. $KMnO_4$ modification.

$KMnO_4$ modification performed in TE buffer at 4°C in the absence (Figure 5A, lane 3) and presence (Figure 5A, lane 4) of 5 mM $MgCl_2$. In Figure 5A, lane 15 1 is the G sequencing reaction; lane 2, is the A+G sequencing reaction.

2. DMS reactivity as a function of temperature.

20 DMS reactivity as a function of temperature was examined in the absence (Figure 5B, lanes 1-5) and presence (Figure 5B, lanes 6-10) of 10 mM $MgCl_2$. Temperatures were as indicated at the top of the figure. Bracketed bases ([]) are affected by $MgCl_2$ concentration, indicated in Figure 5A, or temperature, indicated in Figure 5B. Helical and loop regions are as described above for Figure 2.

E. Reactivity of oligonucleotide V toward $KMnO_4$, DEPC and DMS.

1. $KMnO_4$ reactivity.

$KMnO_4$ reactivity was examined in a buffer containing 25 mM NaOAc and 25 mM Tris-HCl at pH 4.5 (Figure 6A, lane 3), pH 6.0 (Figure 6A, lane 4) and 35 7.0 (Figure 6A, lane 5) at 4°C.

2. DEPC and DMS reactivity.

DEPC (Figure 6B, lanes 3-7) and DMS (Figure 6B, lanes 8-12) reactivity at 4°C in the same buffer; pH and MgCl₂ were as indicated. Bases indicated by closed arrowheads are reactive toward the chemical probes, whereas those indicated by open arrowheads are protected.

3. DEPC reactivity as a function of temperature.

DEPC reactivity in pH 7.0 buffer was examined as a function of temperature in the absence (Figure 6C, lanes 3-6) and presence (Figure 6C, lanes 7-10) of 10 mM MgCl₂. Temperatures were as indicated at the top of the figure. Arrowheads designate adenines whose reactivities are temperature-dependent. In each panel of Figure 6, lane 1 shows the G sequencing reaction and lane 2 shows the A+G sequencing reaction. Helical and loop regions are as described above for Figure 2.

Example 2

Band Shift Assays

Triplex formation is analyzed essentially as described by Roberts et al. (1991).

Figure 9A shows a sample target duplex sequence (SEQ ID NO:6). A representative target sequence to be used for triplex formation is shown in bold; Figure 10A-1 and 10A-2 illustrate the sequences of sample third strand oligonucleotides (SEQ ID NO:9 and SEQ ID NO:10).

The two oligonucleotides labeled A-1 and A-2 both have sequences amenable to triple strand formation. Both oligonucleotides bind in the major groove to the top strand presented in Figure 9A; oligonucleotide A1 is anti-parallel in the major groove and oligonucleotide A2 is parallel in the major groove.

Oligonucleotides are tested for triplex formation in band shift assays on non-denaturing high percentage polyacrylamide gels. Briefly, the two strands of duplex oligonucleotide shown in Figure 9A are each synthesized, and then annealed to form the duplex DNA molecule shown in Figure 9A. Typically the annealing reaction involves mixing the two purified oligonucleotides in 1X TE+50 buffer (10 mM Tris HCl pH 7.4, 1 mM EDTA, 50 mM NaCl), or other suitable buffer (Sauer et al.). The annealing mixture is heated to approximately 85°C and cooled slowly to room temperature. This duplex DNA is then end-labeled using polynucleotide kinase and γ -³²P-ATP (Ausubel et al.) as described above.

The radiolabeled duplex is then mixed individually with each third strand oligonucleotide being tested in 20 mM HEPES, pH 7.1, 50 mM NaCl, 5mM spermine. In general, the oligonucleotide single-strand is added in vast excess (e.g., 10 micromolar) to the ³²P-labeled duplex oligonucleotide (e.g., 1.0 nM). Reaction volumes are typically 15-20 microliters. The samples are then loaded on a 20% acrylamide non-denaturing gel, acrylamide/bisacrylamide 19:1, formed using 20 mM HEPES, pH 7.1 buffer. The running buffer of the gels is (same hepes 20 mM, 7.1. The gels are typically run at 6V/cm at 4°C.

The gels are dried and exposed to X-ray film. The formation of triplex complexes is detected by a band shift, by reduction in the intensity of the duplex band and the appearance of a higher molecular weight band, which contains the duplex complexed to the test oligonucleotide to form the triplex structure.

For each oligonucleotide the concentration of duplex can be held constant and the concentration of the test oligonucleotide varied over a concentration range to give an idea of concentration effects

associated with the test oligonucleotide. Any oligonucleotide which potentially can form triplex structures with a given target duplex, can be tested as described above.

5

Example 3

Modification of Third-Strand Oligonucleotides

Using 1, 10 Phenanthroline

During the chemical synthesis, each oligonucleotide is synthesized with a thiol group at the 5' end using the "C6-THIOL MODIFIER" reagent from Clonetech (Palo Alto, CA), according to the manufacturers instructions. Briefly, the oligonucleotides are deprotected after synthesis with NH_4OH and then
10 treated with silver nitrate to expose the thiol group. The oligonucleotide is immediately reacted with 5-iodoacetamido 1,10-phenanthroline. Commercially available 5-nitro-1,10-phenanthroline (Sigma)
15 is converted to 5-iodoacetamido-1,10-phenanthroline (Chen et al., 1986, herein incorporated by reference) (Figure 11) to covalently link 1,10-phenanthroline to the oligonucleotide.
20

After the addition of the phenanthroline moiety, the resulting oligonucleotides are separated
25 from un-reacted iodo-compound by passing the reaction mixtures through "SEPHADEX G-50" spin columns (Pharmacia, Piscataway NJ).

Phenanthroline attached to an oligonucleotide binds cupric ion and this complex can be used to
30 cleave DNA. In the presence of a reducing agent the bound cupric ion is reduced to cuprous ion, which reduces molecular oxygen to produce hydrogen peroxide. The H_2O_2 reacts with the cuprous complex to form a copperoxo species that is directly responsible for cleavage (Sigman, 1990).
35

An alternative but chemically analogous system (Dreyer et al., 1985; Dervan, 1986; Moser et al.,

1987; Maher et al., 1989) utilizes EDTA-chelated iron tethered to an oligonucleotide to cleave DNA.

The above cleaving agents were also described in co-owned, co-pending U.S. Application Serial No. 5 07/808,452, herein incorporated by reference.

Example 4

In Vitro Cleavage of Target Duplexes

A. pHIV-1LTR-CAT Substrate.

10 To evaluate the ability of the phenanthroline-coupled-oligonucleotide to mediate in vitro cleavage, pHIV-1LTR-CAT (S. Miller, SRI International, Menlo Park CA) is linearized with *HindIII* (Boehringer Mannheim, Indianapolis IN), end-labeled with
15 ^{32}P - γ -ATP (NEN) and polynucleotide kinase, and subjected to a second restriction digest to obtain a uniquely labeled DNA fragment; i.e., one carrying label only at one end of the linearized molecule. The pHIV-1LTR-CAT fragment carrying the target
20 sequence and end-label is gel purified (Ausubel et al.; Sambrook et al.).

After gel purification, this DNA fragment is mixed with phenanthroline modified oligonucleotide in a buffer containing 10 mM Tris-HCl, 100 mM NaCl,
25 500 mM spermine, and 20 mM MgCl_2 . The pH of the buffer is adjusted depending on the sequence of the target (a lower pH is used for the formation of $\text{C}^+\bullet\text{GC}$ base triplets). After incubation at 20°C for 30 min, cleavage is initiated by adding CuSO_4 (to 10 mM)
30 and mercaptopropionic acid (to 2.5 mM). Cleavage products are resolved on sequencing gels along with the products of sequencing reactions for the pHIV-1LTR-CAT target fragment.

B. Alternative Substrates

35 The oligonucleotides shown in Figure 14A are used to evaluate the lengths of purine and pyrimi-

dine tracts which are useful in the practice of the present invention.

The oligonucleotide sequences (Figure 14A) are inserted into pUC18 (Bethesda Research Laboratories, Gaithersburg MD) between the *EcoRI* and *BamHI* sites. The recombinant plasmids are purified and used as substrates for affinity cleaving assay. For the affinity cleaving assay, single-stranded oligonucleotides covalently bound to Cu(II)-1,10-phenanthroline are used as third strands. Two different third strands are possible for each target sequence; both candidates are synthesized and studied. For example, the two oligonucleotides shown in Figure 14B can form triplexes with the duplex target sequence #3 shown in Figure 14A.

The cleavage reactions are carried out and analyzed essentially as described above.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Jayasena, Sumedha D.
Johnston, Brian H.

5

(ii) TITLE OF INVENTION: Triple Helix Formation at
(PuNPyN)-(PuNPyN) Tracts

(iii) NUMBER OF SEQUENCES: 53

10

(iv) CORRESPONDENCE ADDRESS:

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15

(v) COMPUTER READABLE FORM:

20

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

30

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/826,934
(B) FILING DATE: 21-JAN-1992

35

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/808,452
(B) FILING DATE: 13-DEC-1991

(viii) ATTORNEY/AGENT INFORMATION:

40

(A) NAME: Fabian, Gary R.
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(C) REFERENCE/DOCKET NUMBER: P-3141

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45

47

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: Oligonucleotide I, FIGURE 8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAAAAAATT TTTTTTTTTT TTTTTTTTTT TT

32

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: Oligonucleotide II, FIGURE 8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTTTTTTTT TAAAAAAA TTTAAAAA AA

32

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid

48

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Oligonucleotide III, FIGURE 8

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAATTTTGTT AAAAATTGTTG TGGTTTAAAA

15

30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

25

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Oligonucleotide IV, FIGURE 8

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAACCCCTT TTGGGGTTTT CTTCTTTTGG GG

35

32

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45

49

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Oligonucleotide V, FIGURE 8

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGATCTCGT TTGAGATCTC TTTACTCTAG AG

32

10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: SEQUENCE A, FIGURE 9

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTAAAAGAA AAGGGGGGAC TGG

23

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: SEQUENCE B, FIGURE 9

45

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTGGGGACT TTCCAGGGAG GCGT

24

5 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: SEQUENCE C, FIGURE 9

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTGGGCGGG ACTGGGGAGT GGCGAGCCC

29

25 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 10, A-1

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGGGGGAAA AGAAAA

16

51

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- 15 (C) INDIVIDUAL ISOLATE: FIGURE 10, A-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTTCTTTTC CCCCCT

20

16

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- 35 (C) INDIVIDUAL ISOLATE: FIGURE 10, B-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGAGGGACCT TTCAGGGG

40

18

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid

52

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 10, B-2

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCCCTGAAAG GTCCCTCC

18

15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

25

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 10, C-1

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGGGGAGAG GGGAGAGGGG GGG

23

35

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

40

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45

53

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 10, C-2

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCCGCCCTGA CCCCTCACCG CTC

23

10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 10, K

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAAAGAAAAG GGGGGA

16

30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 10, L

45

54

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

18

GGGGATCCCT TAGGGAGG

5 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 10, M

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

26

GGGTGGGATC GGGGAGCGGT GGAGAG

25 (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12A, (D)

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

30

GGTAGAAGAG GAGGAAAAAA GGAAAACTG

55

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 47 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: FIGURE 12, (E)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCCGAAGAAA TAGAAGAAGA AGGTGGAGAG CGAGGAGAGA CAGATCC

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: FIGURE 12A, (F)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCACTTTTAA AAGAAAAGG GGGGACTGGA AGGGCTA

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid

56

- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, D-1

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCTTCTCCTC CTTTTTCCT TTTNNNNNT TTTTCCTTTT TTCCTCCTCT TCT

53

15

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53 base pairs

20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

25

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, D-2

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGAAGAGGAG GAAAAAAGGA AAAANNNNT TTTTCCTTTT TTCCTCCTCT TCT

53

35

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87 base pairs

40

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45

57

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, E-1

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTTCTTTATC TTCTTCTTCC ACCTCTCGCT CCTCTCTGTC TNNNNNTCTG TCTCTCCTCG 60

10

CTCTCCACCT TCTTCTTCTA TTTCTTC 87

87

(2) INFORMATION FOR SEQ ID NO:24:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, E-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

30

GAAGAAAAG AAGAAGAAGG AGGAGAGGGA GGAGAGAGAG ANNNNTCTG TCTCTCCTCG 60

60

CTCTCCACCT TCTTCTTCTA TTTCTTC 87

87

35 (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 base pairs

(B) TYPE: nucleic acid

40

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45

(iii) HYPOTHETICAL: NO

58

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, F-1

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GAAAAATTTT CTTTTCCTCC CTCTCCTTCC CNNNNNCCCT TCCAGTCCCC CCTTTTCTTT 60

TTTTTTC 67

10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, F-2

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAAAAAAGG GAAAGGGGG GAGAGGAAGG GNNNNNCCCT TCCAGTCCCC CCTTTTCTTT 60

30

TAAAAAG 67

(2) INFORMATION FOR SEQ ID NO:27:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

59

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, Dx

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCTTCTCCTC CTTTTTCCT TTTT

24

(2) INFORMATION FOR SEQ ID NO:28:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, Ex

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTTCTTTATC TTCTTCTCC ACCTCTCGCT CCTCTCTGTC T

41

(2) INFORMATION FOR SEQ ID NO:29:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

40

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 12B, Fx

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTTTTTTTTT CTTTCCCCC CTGACCTCC C

31

5 (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #1

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AATTCGGGGG GGGGGTTTTT TTTTGG

26

25 (2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #1

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCCCCCCCCC CAAAAA AAAA ACCTAG

26

61

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- 15 (C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AATTCGGGGG GTTTTTTTTG GGGGGG

20

26

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- 35 (C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCCCCC AAAA ACCCCC CCCTAG

40

26

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid

62

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #3

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AATTCGGGGG TTTTGGGGG TTTTG

26

15

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

25

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #3

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCCCCCAAAA ACCCCCAAAA ACCTAG

26

35

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

40

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (g nomic)

45

63

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #4

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AATTCGGGGT TTTGGGGTTT TGGGGG

26

10

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #4

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCCCCAAAAC CCCAAAACCC CCCTAG

26

30

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #5

45

64

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

AATTCGGGTT TTGGGTTTGG GTTTGG

26

5 (2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #5

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GCCCCAAACC CAAACCCAAA ACCTAG

26

25 (2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #6

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AATTCGGGTT TGGGTTTGGG TTTGGG

26

65

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- 15 (C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

20 GCCCAAACCC AAACCCAAAC CCCTAG

26

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- 35 (C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

40 AATTCGGTTG GTTGTTGGT TGGTTG

26

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid

66

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #7

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCCAACCAAC CAACCAACCA ACCTAG

26

15

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

25

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #8

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

AATTCGTGTG TGTGTGTGTG TGTGTG

26

35

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: DNA (genomic)

45

67

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14A, TARGET SEQUENCE #8

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GCACACACAC ACACACACAC ACCTAG

26

10

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14B

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TTTTTGGGGG TTTTTGGGGG

20

30

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 14B

45

68

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCCCCAAAAA CCCCCAAAAA

20

5 (2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 15A

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GGGGGTTTTT GGGGGTTTTT

20

25 (2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 15B, I1

40

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 6..10

(D) OTHER INFORMATION: /note= "WHERE N IS INOSINE"

69

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 16..20

(D) OTHER INFORMATION: /note= "WHERE N IS INOSINE"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TTTTTNNNNN TTTTTNNNNN

20

10

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 15B, I2

25

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 6..10

(D) OTHER INFORMATION: /note= "WHERE N IS INOSINE"

30

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 16..20

(D) OTHER INFORMATION: /note= "WHERE N IS INOSINE"

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CCCCCNNNNN CCCCCNNNNN

20

40

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

45 (B) TYPE: nucleic acid

70

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 15B, G1

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TTTTTGGGGG TTTTGGGGG

20

15

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

20

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

30

(C) INDIVIDUAL ISOLATE: FIGURE 15B, A1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

35 CCCCCAAAAA CCCCCAAAAA

20

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

40

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: DNA (genomic)

71

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: FIGURE 15A

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AAAAATTTTT AAAAATTTTT

20

IT IS CLAIMED:

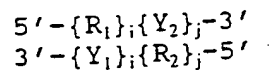
1. An oligonucleotide capable of forming a triple-helix with a duplex nucleic acid molecule, which contains a target nucleic acid sequence, where said nucleic acid has first and second complementary strands and said first strand has a target sequence with at least one tract of purines adjacent at least one tract of pyrimidines, and said oligonucleotide contains at least one pyrimidine tract adjacent one purine tract, and where either

(i) the pyrimidine tract is parallel to the purine tract of the first strand target sequence and the purine tract is antiparallel to the purine tract of the second strand target sequence, or

(ii) the purine tract is antiparallel to the purine tract of the first strand target sequence and the pyrimidine tract is parallel to the purine tract of the second strand,

and where the oligonucleotide pyrimidine and purine tracts have nucleic acid sequences which can pair, with the purine strands of the target sequence of the duplex, to form a triple helix.

2. The oligonucleotide of claim 1, wherein the target sequence of the duplex nucleic acid comprises

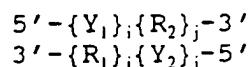


where R_1 and Y_1 are, respectively, complementary tracts of purines and pyrimidines, R_2 and Y_2 are, respectively, complementary tracts of purines and pyrimidines, and i and j are greater than or equal to 4,

and where said oligonucleotide contains a sequence selected from the group consisting of $3'-\{r_1\}_i\{y_2\}_j-5'$ and $5'-\{Y_1\}_i\{R_2\}_j-3'$,

where r is A or T for $R=A$ and r is G for $R=G$, and y is T when $Y=A$ and y is C when $Y=G$.

3. The oligonucleotide of claim 1, wherein the target sequence of the duplex nucleic acid comprises



5 where R_1 and Y_1 are, respectively, complementary tracts of purines and pyrimidines, R_2 and Y_2 are, respectively, complementary tracts of purines and pyrimidines, and i and j are greater than or equal to 4,

10 and where said oligonucleotide contains a sequence selected from the group consisting of $5'-\{r_1\}_i\{y_2\}_j-3'$ and $3'-\{y_1\}_i\{r_2\}_j-5'$,

where r is A or T for $R=A$ and r is G for $R=G$, and y is T when $Y=A$ and y is C when $Y=G$.

15

4. The oligonucleotide of claim 1, wherein said target nucleic acid sequence contain one purine tract adjacent at least one pyrimidine tract.

20

5. The oligonucleotide of claim 1, wherein the target sequence contains at least about 8 nucleotides.

25

6. The oligonucleotide of claim 1, wherein the nucleic acid molecule is a duplex DNA molecule.

30

7. The oligonucleotide of claim 1, which further includes at least one moiety attached to the oligonucleotide, where the moiety is capable of cleaving the DNA molecule containing the target nucleic acid sequence.

35

8. The oligonucleotide of claim 7, wherein the cleaving moiety is selected from the group consisting of phenanthroline Cu(II), Zn(II), Fe(II)-EDTA, Cu(II)-bipyridine, and Cu(II)-terpyridine..

9. A method of cleaving a duplex nucleic acid molecule, having first and second complementary strands, containing a target nucleic acid sequence, where said first strand has a target sequence with
5 at least one tract of purines adjacent at least one tract of pyrimidines, comprising

contacting the nucleic acid molecule with an oligonucleotide, where said oligonucleotide contains at least one pyrimidine tract adjacent one purine
10 tract, and where either

(i) the pyrimidine tract is parallel to the purine tract of the first strand target sequence and the purine tract is antiparallel to the purine tract of the second strand target sequence, or

15 (ii) the purine tract is antiparallel to the purine tract of the first strand target sequence and the pyrimidine tract is parallel to the purine tract of the second strand,

and where the oligonucleotide pyrimidine and
20 purine tracts have nucleic acid sequences which can pair, with the purine strands of the target sequence of the duplex, to form a triple helix,

said oligonucleotide further includes at least one moiety which is capable of cleaving the nucleic
25 acid molecule containing the target nucleic acid sequence, and

where said contacting is at an oligonucleotide concentration effective to produce cleavage of the nucleic acid molecule containing the target nucleic
30 acid sequence.

10. The method of claim 9, wherein said contacting further includes a reducing agent selected from the group consisting of mercaptopropionic
35 acid and ascorbate.

11. The method of claim 9, wherein said moiety is selected from the group consisting of phenanthroline Cu(II), Zn(II), Fe(II)-EDTA, Cu(II)-bipyridine, and Cu(II)-terpyridine.

5

12. The method of claim 11, wherein cleaving moiety is phenanthroline.

13. The method of claim 12, wherein said
10 contacting further includes the addition of CuSO₄ and mecaptopropionic acid.

14. The method of claim 9, wherein the nucleic
acid molecule containing the target nucleic acid
15 sequence is a duplex DNA molecule.

15. An oligonucleotide containing three
regions capable of forming a triple-helix with a
single-strand nucleic acid molecule, which contains
20 a target nucleic acid sequence with at least one
tract of purines adjacent at least one tract of
pyrimidines, and said oligonucleotide contains (i)
in the first region a nucleic acid sequence comple-
mentary to the nucleic acid sequence of the target
25 nucleic acid effective to form a duplex nucleic
acid, (ii) in the second region, a stable abasic
connecting sequence, and (iii) in the third region
at least one pyrimidine tract in parallel to one
purine tract on either strand of the duplex nucleic
30 acid, adjacent at least one purine tract in anti-
parallel to the purine tract on the opposite strand
of the duplex nucleic acid,

and where the oligonucleotide pyrimidine and
purine tracts in the third region have nucleic acid
35 sequences which can pair, with the purine strands of
the target sequence of the duplex, to form a triple
helix.

16. The oligonucleotide of claim 15, wherein the nucleic acid molecule is a single stranded DNA molecule.

5 17. The oligonucleotide of claim 15, wherein the nucleic acid molecule is an RNA molecule.

10 18. The oligonucleotide of claim 15, which further includes at least one moiety attached to the oligonucleotide, where the moiety is capable of cleaving the nucleic acid molecule containing the target nucleic acid sequence.

15 19. A method of cleaving a single-strand nucleic acid molecule containing a target nucleic acid sequence, where said target sequence has at least one tract of purines adjacent at least one tract of pyrimidines, comprising

20 contacting the nucleic acid molecule with an oligonucleotide, having three regions, comprised of (i) in the first region a nucleic acid sequence complementary to the nucleic acid sequence of the target nucleic acid effective to form a duplex nucleic acid, (ii) in the second region, a stable

25 abasic connecting sequence, and (iii) in the third region at least one pyrimidine tract in parallel to one purine tract on either strand of the duplex nucleic acid, adjacent at least one purine tract in antiparallel to the purine tract on the opposite

30 strand of the duplex nucleic acid, where the oligonucleotide pyrimidine and purine tracts in the third region have nucleic acid sequences which can pair, with the purine strands of the target sequence of the duplex, to form a triple helix,

35 and where said oligonucleotide includes at least one moiety which is capable of cleaving the

nucleic acid molecule containing the target nucleic acid sequence,

where said contacting is at an oligonucleotide concentration effective to produce cleavage of the nucleic acid molecule containing the target nucleic acid sequence.

20. The method of claim 19, wherein the nucleic acid molecule is a single stranded DNA molecule.

21. The method of claim 19, wherein the nucleic acid molecule is an RNA molecule.

22. The method of claim 21, wherein the RNA molecule is the HIV-1 genome.

23. The method of claim 22, wherein the oligonucleotide consists essentially of a sequence selected from the group of sequences presented as SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26.

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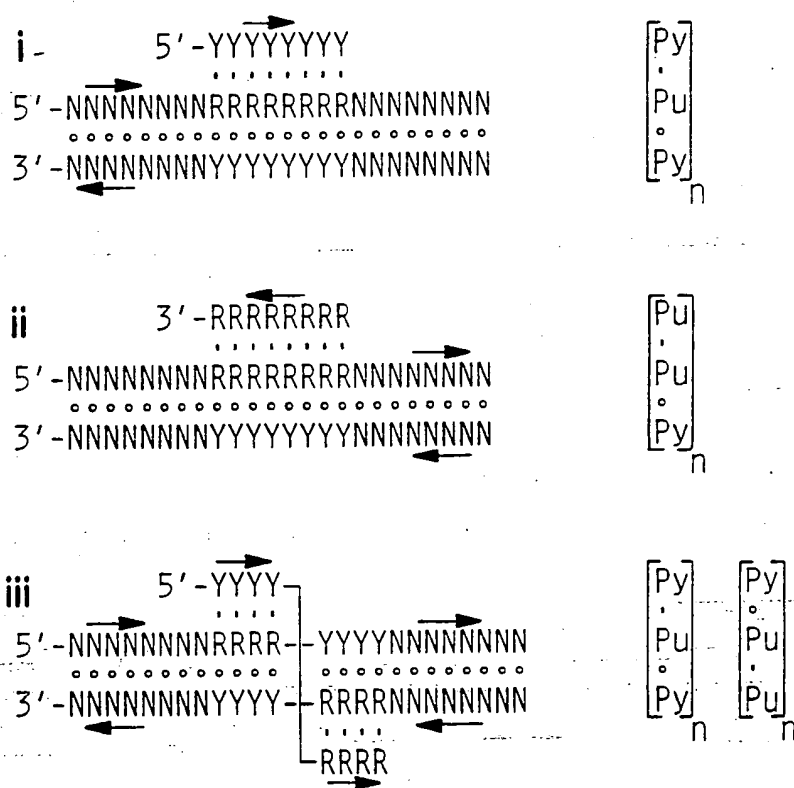


FIG. 1a

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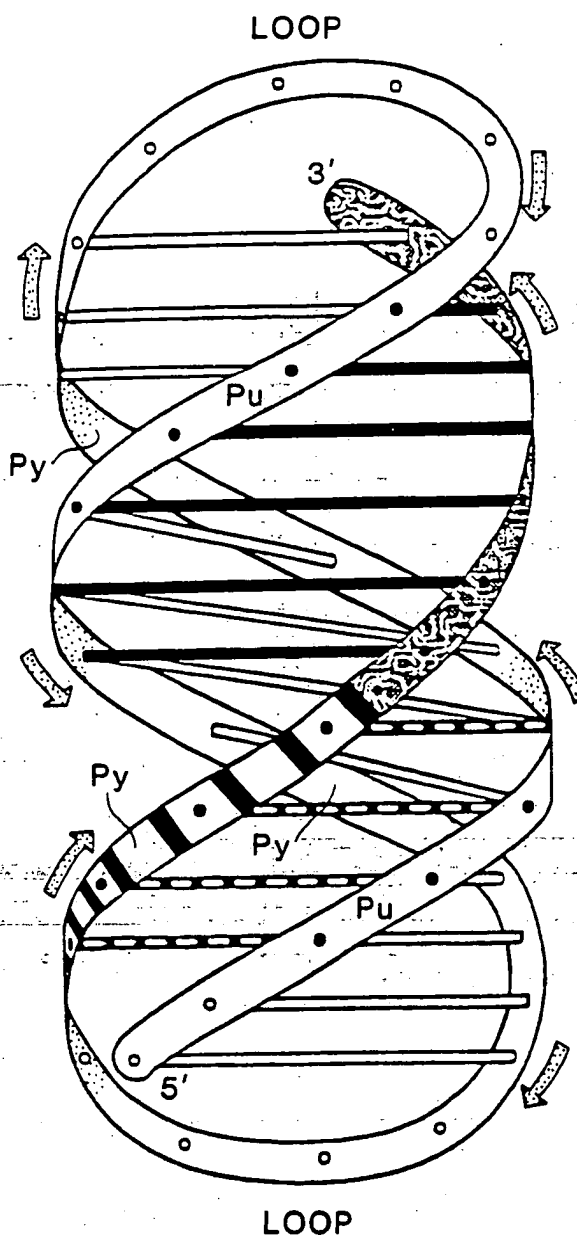


FIG. 1b

SUBSTITUTE SHEET

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R. - KMnO_4 -
 Mg^{++} (mM) 0 5 10 20
1 2 3 4 5

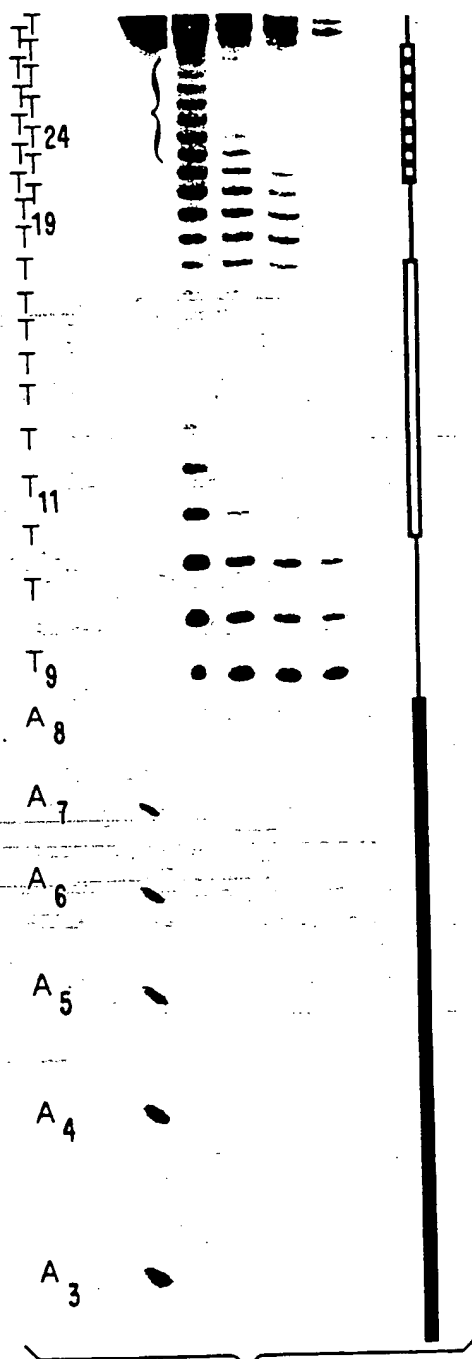


FIG. 2

SUBSTITUTE SHEET

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	R KMnO ₄				R DEPC			
Mg ⁺⁺ (mM)	0	5	10		0	5	10	
	1	2	3	4	5	6	7	8

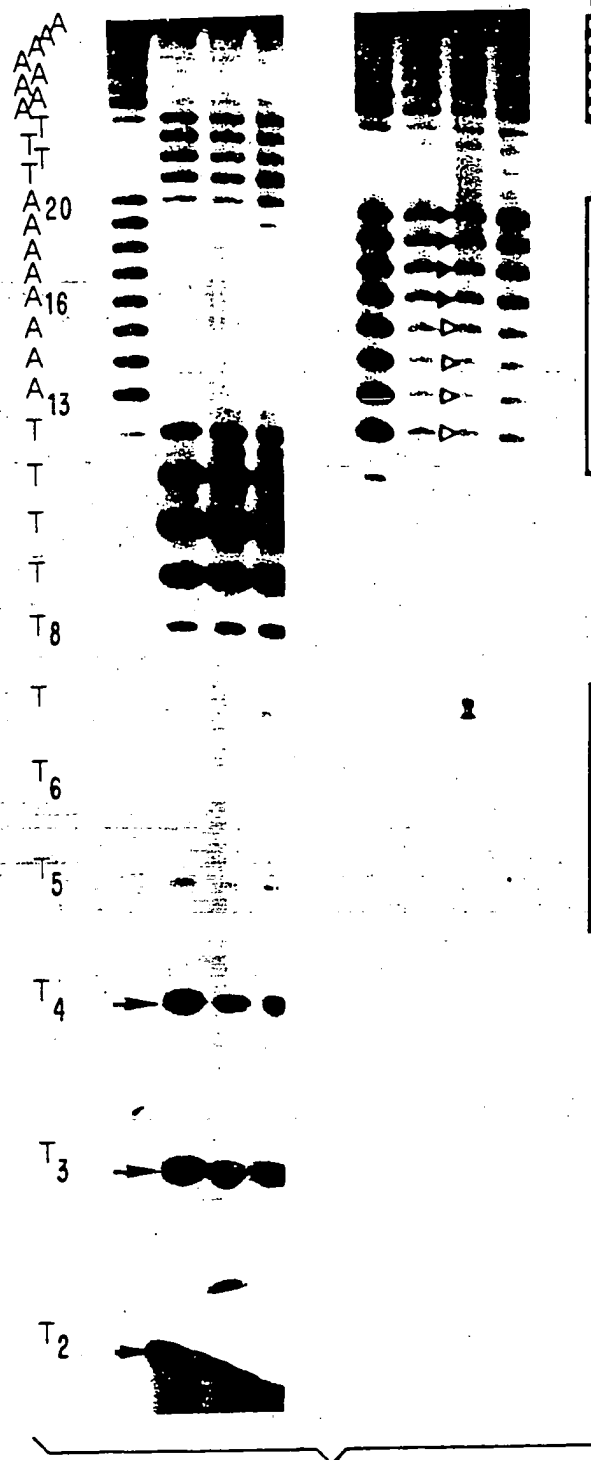


FIG. 3

SUBSTITUTE SHEET

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	G R - KMnO ₄ -						G R - DEPC -					
Mg ⁺⁺ (mM)	0 5 10 20						0 5 10 20					
	1	2	3	4	5	6	7	8	9	10	11	12

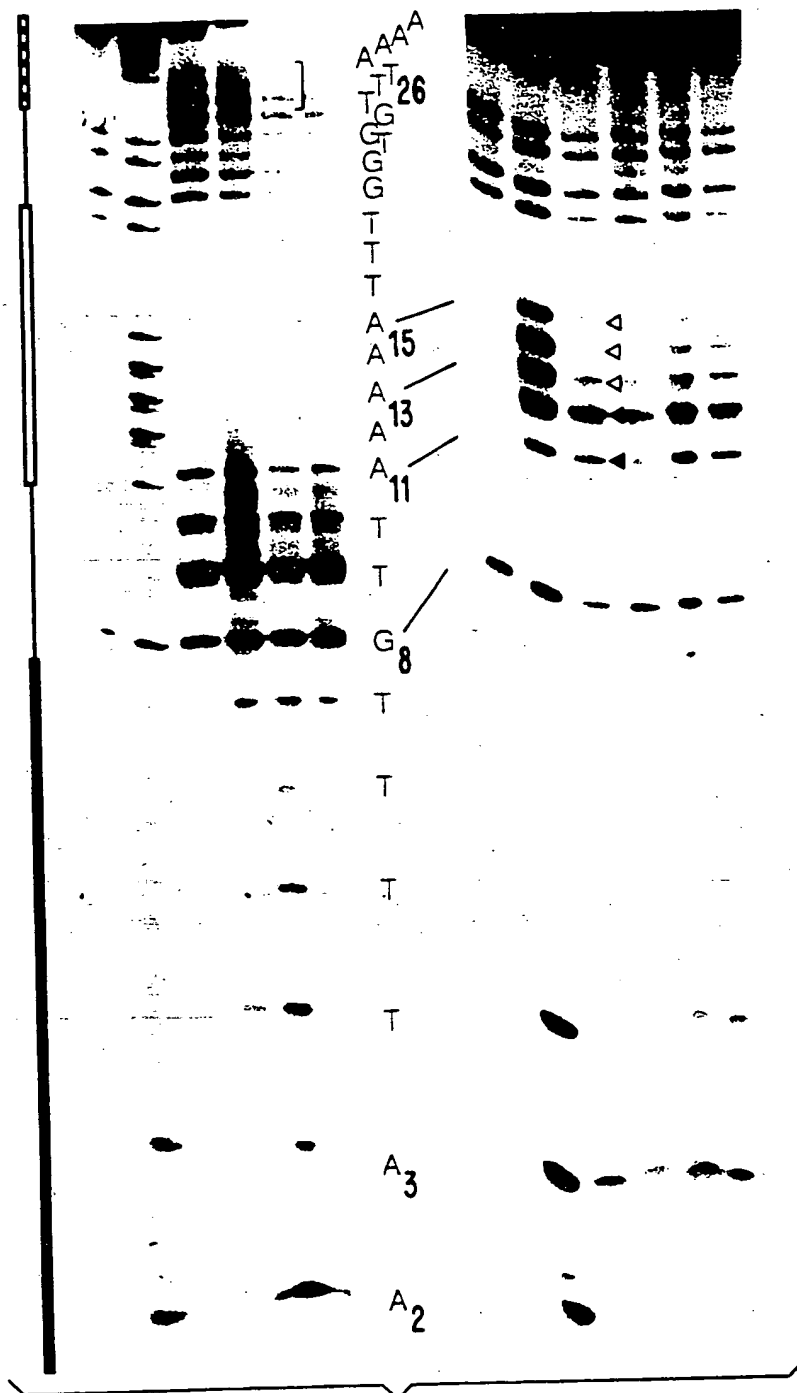


FIG. 4a

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	DEPC									
Mg ⁺⁺ (mM)	0					10				
T(°C)	4	23	37	55	70	4	23	37	55	70
	1	2	3	4	5	6	7	8	9	10



A₃



A₂



FIG. 4b

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FIG. 5a

G R KMnO₄

Mg⁺ (mM) 0 5

T (°C) -4 -

1 2 3 4

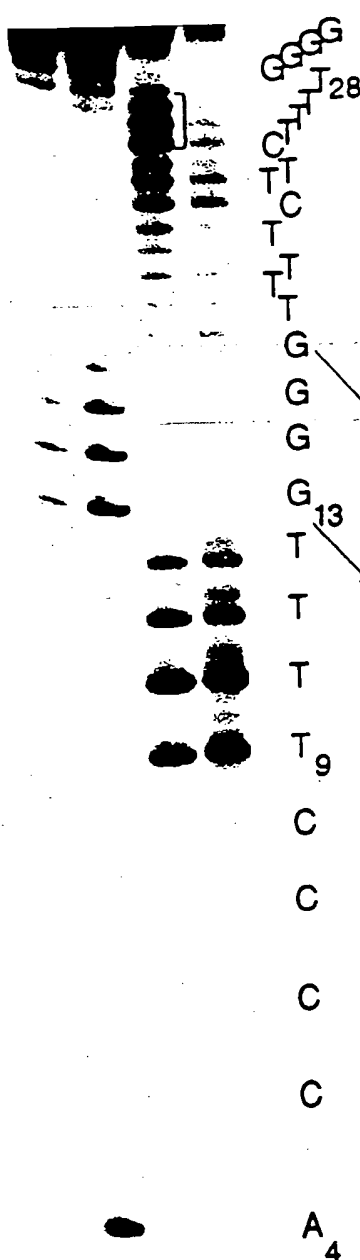


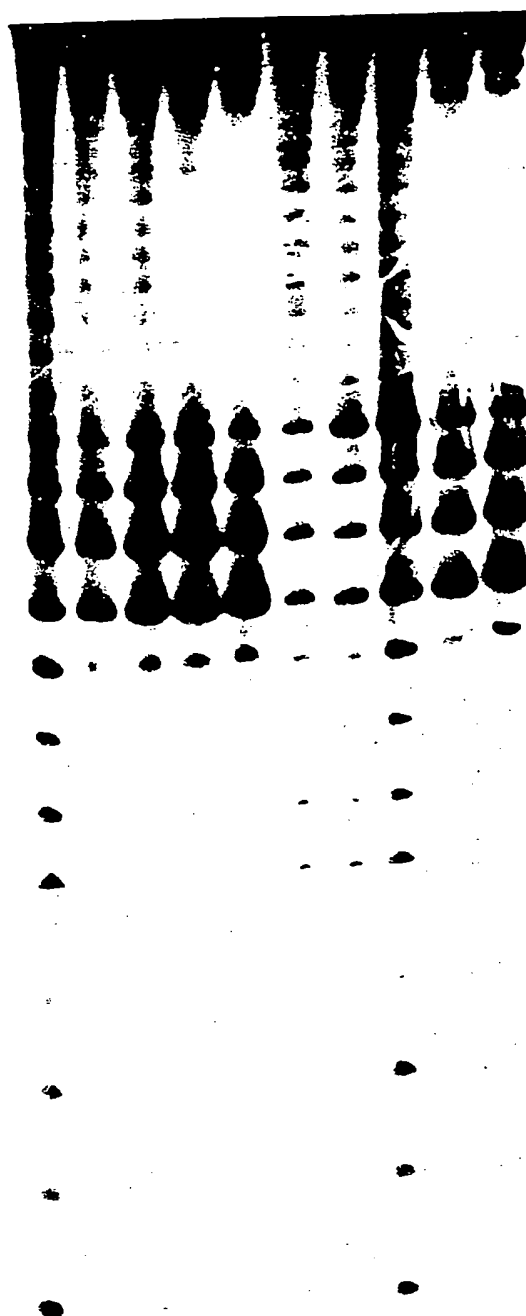
FIG. 5b

———— DMS ————

———— 0 ———— | ———— 10 ————

4 23 37 55 70 4 23 37 55 70

1 2 3 4 5 6 7 8 9 10



SUBSTITUTE SHEET

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FIG. 6a

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	G		R		— DEPC —						I		— DMS —							
			4.5			7.0			7.0			4.5								
pH																				
Mg ²⁺ (mM)			0			10			20			0			10			20		
	1	2	3	4	5	6	7	8	9	10	11	12								

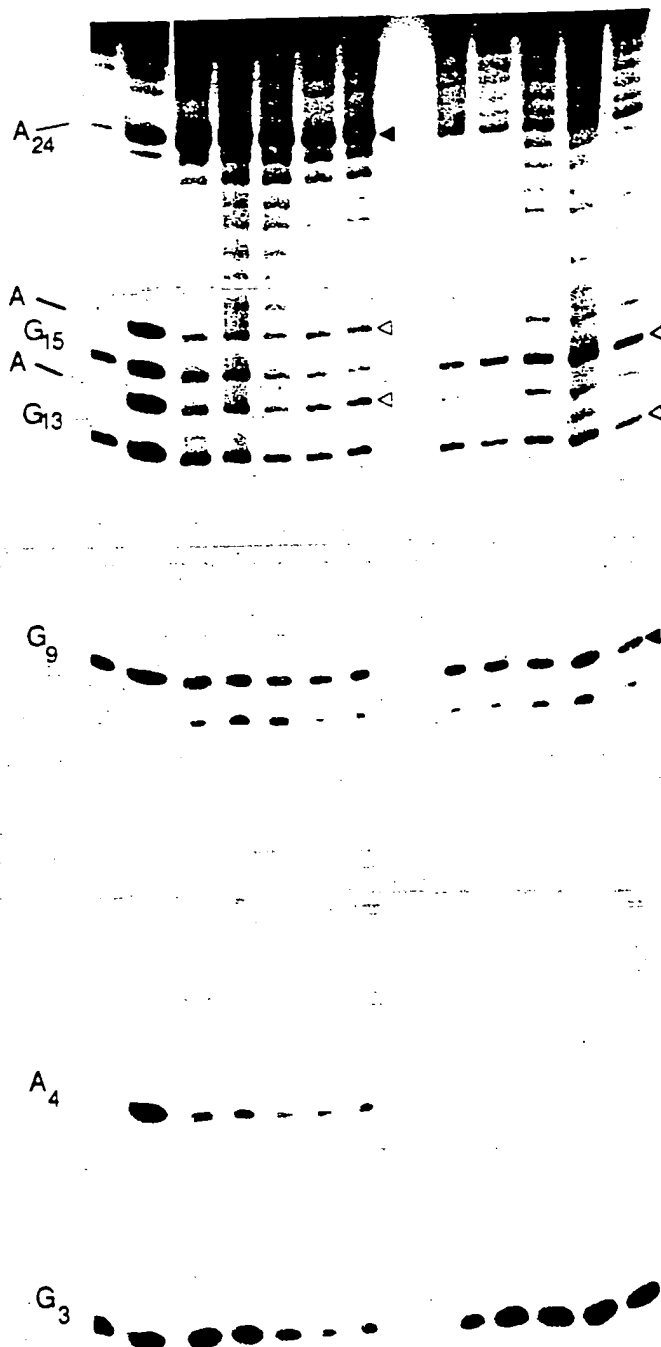


FIG. 6b

SUBSTITUTE SHEET

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	G	R	DEPC							
Mg ⁺⁺ (mM)	0	1	10							
T(°C)	23	37	55	70	23	37	55	70		
	1	2	3	4	5	6	7	8	9	10

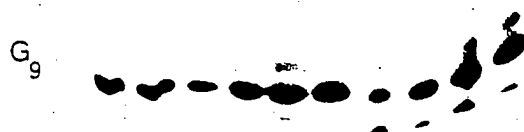
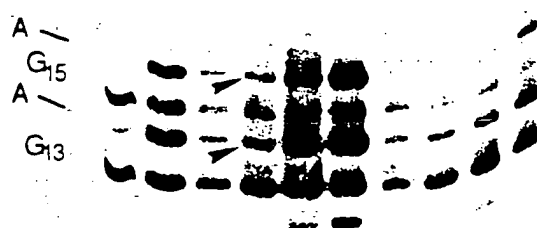
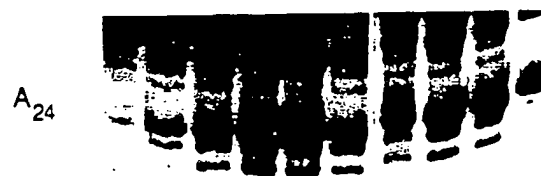
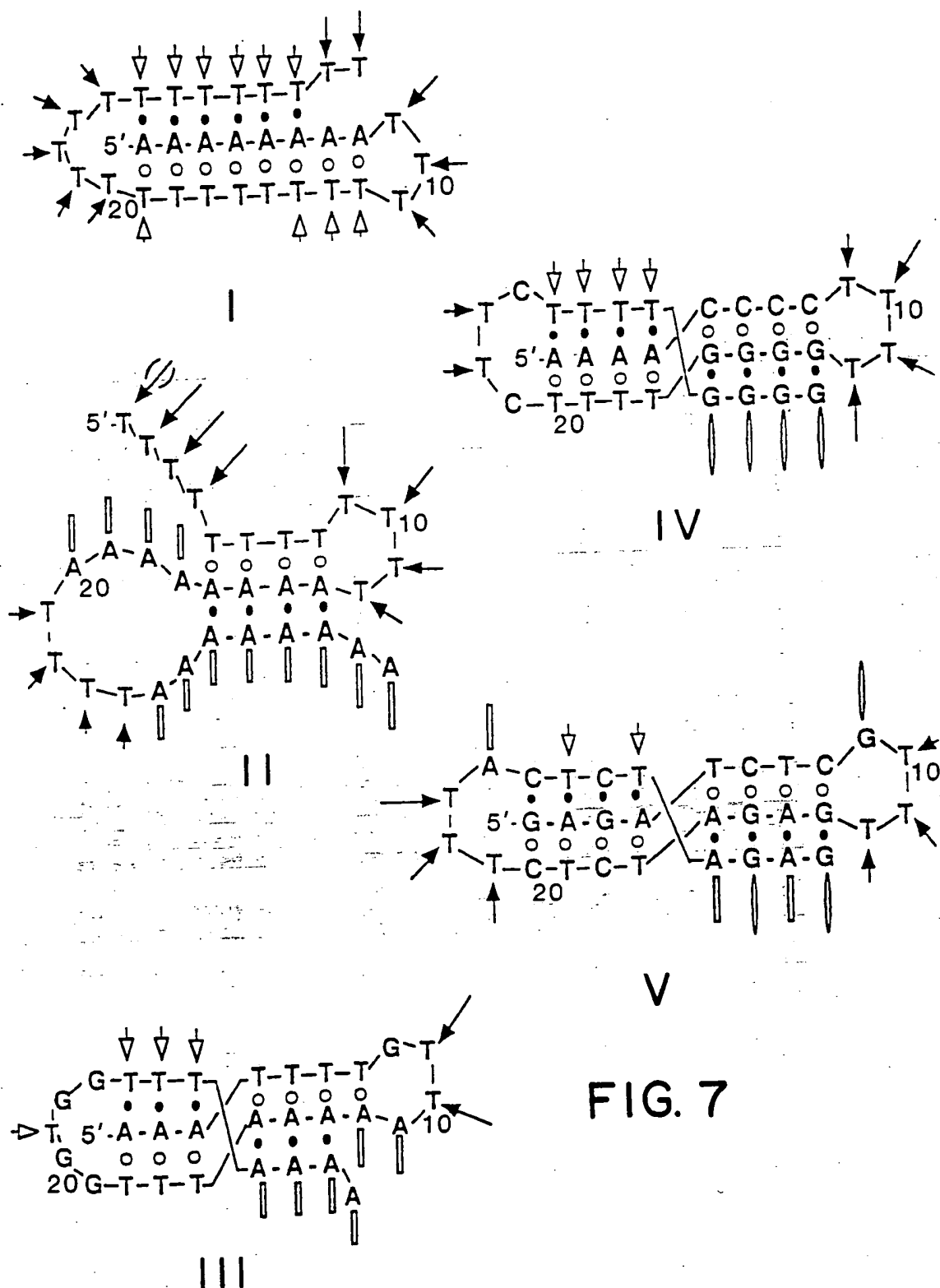


FIG. 6c

SUBSTITUTE SHEET

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FIG. 8

I 5'-AAAAAAATTTT'TTTT'TTTT'TTTT'TTTT'TTTT'-3'
II 5'-TTT'TTT'TTTTAAATAAAATTTTAAATAAA-3'
III 5'-AAATTTGTAAATAATTGGTGGTTTAAAA-3'
IV 5'-AAACCCCTTTTGGGGTTTCTTCTTTTGGGG-3'
V 5'-GAGATCTCGTTTGAGATCTCTTTACTCTAGAG-3'

FIG. 9

5'-TTTAAAGAAAGGGGGACTGG
AAATTTCTTTTCCCCCTGACC-5'

5'-GCTGGGGACTTCCAGGGAGGCGT
CGACCCCTGAAGGTCCCTCCGCA-5'

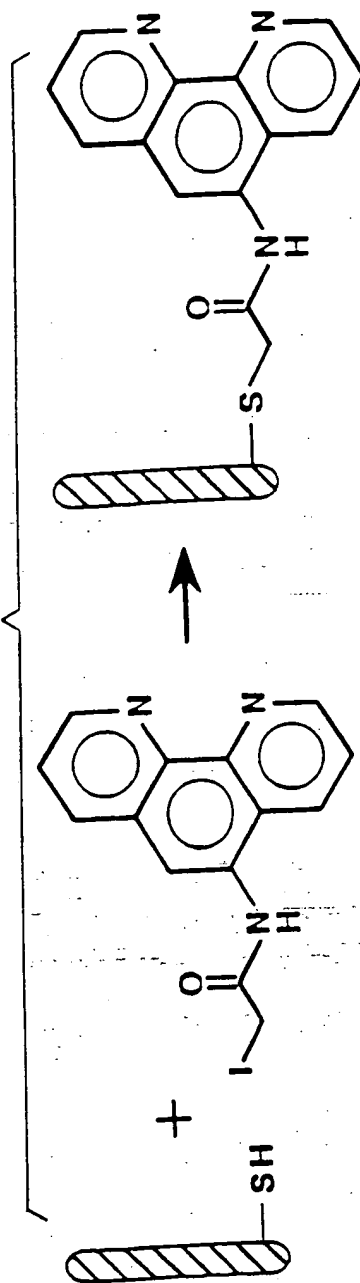
5'-CCTGGGGGGACTGGGGAGTGGCGAGCCC
GGACCCGCCCTGACCCCTCACCGCTCGGG-5'

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A-1	5'-AGGGGGGAAAAGAAA	Pu•PuPy
A-2	5'-TTTTCTTTTCCCCCT	Py•PuPy
B-1	5'-GGAGGGACCTTTCAGGG	Pu•PuPy - Py•PuPy - Pu•PuPy
B-2	5'-CCCCTGAAAGGTCCCTCC	Py•PuPy - Pu•PuPy - Py•PuPy
C-1	5'-GAGGGGAGGGGAGGGGGG	Pu•PuPy
C-2	5'-CCCGCCCTGACCCCTCACCGCTC	Pu•PuPy >>Py•PuPy
K	5'-AAAAGAAAAGGGGGA	
L	5'-GGGGATCCCTTAGGGAGG	
M	5'-GGGTGGGATCGGGAGCGGTGGAGAG	

FIG. 10

FIG. 11



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6184 5'-GGTAGAAGAGGAGGAAAAAAGGAAAAACTG (D)

7916 5'-CCCGAAGAAATAGAAGAAGAAGGTGGAGAGCGAGGAGAGACAGATCC (E)

5867 5'-CCACTTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTA (F)

FIG. 12a

D-1 5'-TCTTCTCCTCCTTTTTTCTTTTT -X-X
 5'-GGTAGAAGAGGAGGAAAAAAGGAAAAACTG I
 3'-TCTTCTCCTCCTTTTTTCTTTTT -X-X

D-2 3'-AGAAGAGGAGGAAAAAAGGAAAAA -X-X
 5'-GGTAGAAGAGGAGGAAAAAAGGAAAAACTG I
 5'-TCTTCTCCTCCTTTTTTCTTTTT -X-X

Dx 3'-TCTTCTCCTCCTTTTTTCTTTTT

E-1 5'-CTTCTTTATCTTCTTCTTCCACCTCTCGCTCCTCTGTCT -X-X
 5'-CCCGAAGAAATAGAAGAAGAAGGTGGAGAGCGAGGAGAGACAGATCC I
 3'-CTTCTTTATCTTCTTCTTCCACCTCTCGCTCCTCTGTCT -X-X

E-2 3'-GAAGAAAAAGAAGAAGAAGGAGGAGAGGGAGGAGAGAGAGA -X-X
 5'-CCCGAAGAAATAGAAGAAGAAGGTGGAGAGCGAGGAGAGACAGAGATCC I
 5'-CTTCTTTATCTTCTTCTTCCACCTCTCGCTCCTCTGTCT -X-X

Ex 3'-CTTCTTTATCTTCTTCTTCCACCTCTCGCTCCTCTGTCT

F-1 3'-GAAAAATTTTCTTTTTCCCCCTCACCTTCCC -X-X
 5'-CCACTTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTA I
 5'-CTTTTTTTTTCTTTTTCCCCCTGACCTTCCC -X-X

F-2 5'-GAAAAAAAAGAAAAGGGGGGAGAGGAAGGG -X-X
 5'-CCACTTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTA I
 3'-GAAAAATTTTCTTTTTCCCCCTGACCTTCCC -X-X

Fx 3'-CTTTTTTTTTCTTTTTCCCCCTGACCTTCCC

FIG. 12b

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AATTCGGGGGGGGGGTTTTTTTTTTG Target sequence #1
GCCCCCCCCCAAAAAAAAAACCTAG

AATTCGGGGGGTTTTTTTTTGGGGGGG Target sequence #2
GCCCCCAAAAAAAAAACCCCCCCTAG

AATTCGGGGGTTTTTGGGGGTTTTTG Target sequence #3
GCCCCCAAAACCCCCAAAAACCTAG

AATTCGGGGTTTTTGGGGTTTTGGGGG Target sequence #4
GCCCCAAAACCCCCAAAACCCCCCTAG

AATTCGGGTTTTGGGTTTGGGTTTTG Target sequence #5
GCCCCAAAACCCAAACCCAAAACCTAG

AATTCGGGTTTGGGTTTGGGTTTGGG Target sequence #6
GCCCCAAACCCAAACCCAAACCCCTAG

AATTCGGTTGGTTGGTTGGTTGGTTG Target sequence #7
GCCAACCAACCAACCAACCAACCTAG

AATTCGTGTGTGTGTGTGTGTGTGTG Target sequence #8
GCACACACACACACACACACCTAG

FIG. 14a**SUBSTITUTE SHEET**

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5'-TTTTTGGGGGTTTTTGGGGG

5'-CCCCCAAAAACCCCCAAAAA

FIG. 14b

GGGGGTTTTTGGGGGTTTTT

CCCCCAAAAACCCCCAAAAA

AAAAATTTTTAAAAATTTTT

TTTTTAAAAATTTTTAAAAA

FIG. 15a

11 5'-TTTTTIIIIIIIIIIIIIIIIII

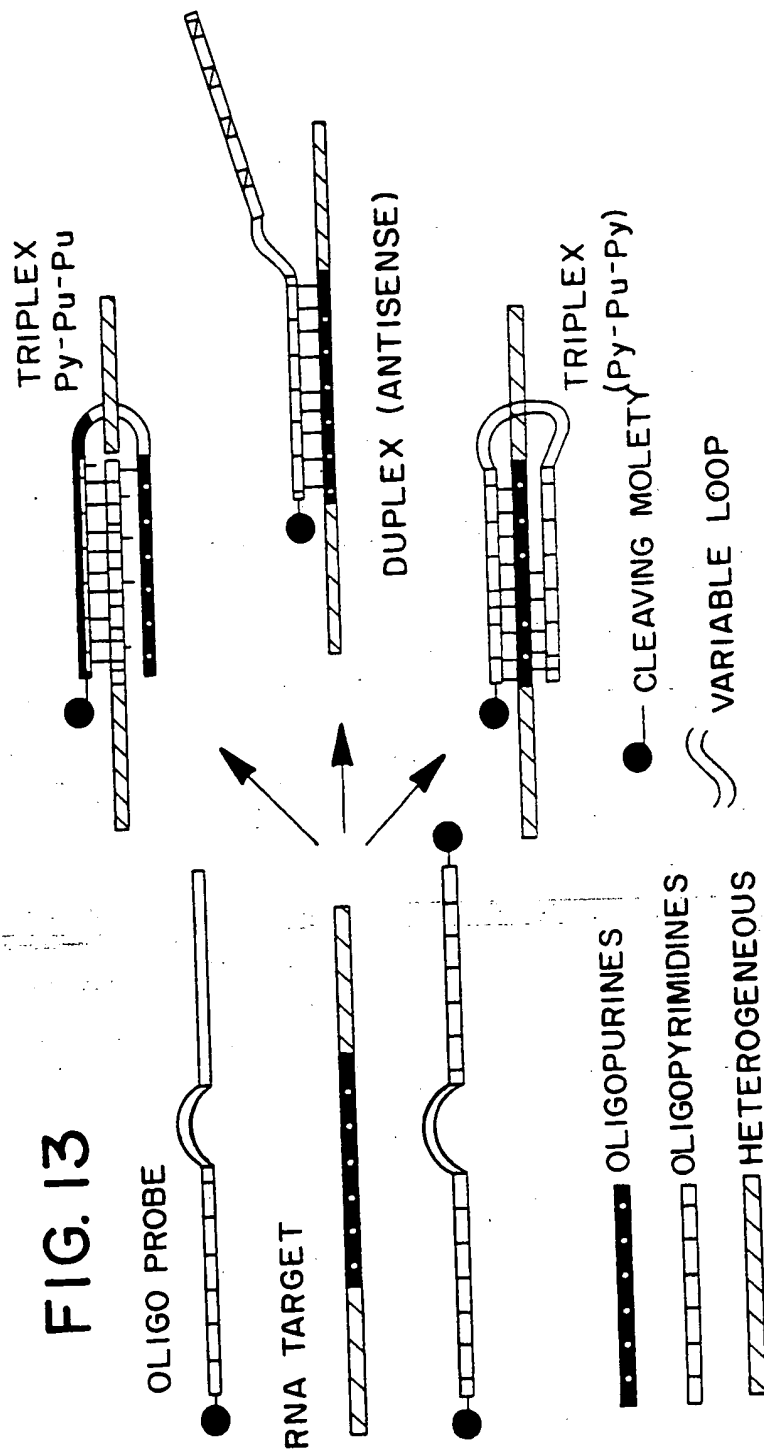
G1 5-TTTTTGGGGGTTTTTGGGGG

12 5'-CCCCCIIIIIIIIIIIIIIIIII

A1 5-CCCCCAAAAACCCCCAAAAA

FIG. 15b**SUBSTITUTE SHEET**

FIG. 13



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/10792

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5 C 12 N 15/11 C 07 H 21/04 A 61 K 31/70 A 61 K 31/44 A 61 K 33/30 A 61 K 31/495		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl.5	C 12 N C 07 H A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9106626 (GILEAD SCIENCES, INC.) 16 May 1991, see page 5, line 22 - page 6, line 7; page 30, lines 7-22; claims 16-18, 22-24	1-3, 2A, 3A, 4
Y		1-3, 2A, 3A, 4, 8- 16, 18- 20, 22- 23
X	--- WO,A,9118997 (GILEAD SCIENCES, INC.) 12 December 1991, see page 4, lines 14-33; page 8, lines 4-22; page 17, lines 17-27	1-3, 2A, 3A, 4, 8, 10, 15 23
Y	--- -/-	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
31-03-1993		27. 04. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		S.M. ANDRES

(CONTINUED FROM THE SECOND SHEET)

Relevant to Claim No.

III. DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to Claim No.
Category	Citation of Document, with indication, where appropriate, of the relevant passages	
Y	Journal of the American Chemical Society, vol. 112, no. 6, 1990, (Washington, DC, US), D.A. HORNE et al.: "Recognition of mixed-sequence duplex DNA by alternate-strand triple-helix formation", pages 2435-2437, see the whole document (cited in the application)	1-3,2A, 3A,4,8-10,12, 15-16, 19-20
Y	--- Nucleic Acids Research, vol. 16, no. 24, 1988, (Arlington, Virginia, US), J.-C. FRANCOIS et al.: "Sequence-specific recognition of the major groove of DNA by oligodeoxynucleotides via triple helix formation. Footprinting studies", pages 11431-11440, see the whole document (cited in the application)	11,13-14
Y	--- Journal of the American Chemical Society, vol. 113, no. 1, 2 January 1991, (Washington, DC, US), A.S. MODAK et al.: "Toward chemical ribonucleases. 2. Synthesis and characterization of nucleoside-bipyridine conjugates. Hydrolytic cleavage of RNA by their copper(II) complexes", pages 283-291, see abstract (cited in the application)	18,22
A	--- Science, vol. 245, 18 August 1989, (Lancaster, Pa, US), L.J. MAHER III et al.: "Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation", pages 725-730, see the whole document (cited in the application)	1,4,8-10,12, 15
A	--- Proceedings of the National Academy of Sciences of USA, vol. 86, December 1989, (Washington, DC, US), J.-C. FRANCOIS et al.: "Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline-copper chelate", pages 9702-9706, see the whole document (cited in the application)	8-9,12
A	--- -	1,4,8-15

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Biochemistry, vol. 31, no. 2, 1992, (Easton, PA, US), S.D. JAYASENA et al.: "Intramolecular triple-helix formation at (PunPyn)(PunPyn) tracts: recognition of alternate strands via Pu.PuPy and Py.PuPy base triplets", pages 320-327, see the whole document ---	1-3,2A, 3A,4
P,X	Nucleic Acids Research, vol. 20, no. 20, 1992, (Arlington, Virginia, US), S.D. JAYASENA et al.: "Oligonucleotide-directed triple helix formation at adjacent oligopurine and oligopyrimidine DNA tracts by alternate strand recognition", pages 5279-5288, see the whole document ---	1-3,2A, 3A,4,8- 15
P,X	Comptes Rendus des Seances de l'Academie des Sciences. Serie III: Sciences de la Vie, vol. 313, no. 13, 19 December 1991, (Montreuil, FR), J.-S. SUN et al.: "Extension of the range of recognition sequences for triple helix formation by oligonucleotides containing guanines and thymines", pages 585-590, see the whole document -----	1-3,2A, 3A,4

INTERNATIONAL SEARCH REPORT

national application No.

PCT/US 92/ 10792

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see attached sheet
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

OBSCURITIES

The set of claims filed with the underlying application contains inconsistencies in the numbering of the claims. Indeed, the first three claims are numbered from 1 to 3 and are followed by another three claims numbered 2, 3 and 4!!! This results in two set of claims having number 2 or 3. The more, the numbering of the claims is interrupted between claim 4 and claim 8 (no claims 5, 6 or 7), which causes lack of ready comprehensibility as regard to claim 9 which refers itself to (a non-existent) claim 7.

Therefore, the Search Authority decided to refer to the duplicated claims 2 and 3 as claims 2A and 3A. The set of claims is therefore to be considered as having the following numbering:

claims 1 to 3, 2A, 3A, 4, 8 to 24 (claims 5 to 7 are inexistant)

The more, the reference to claim 7 found in claim 9 was assumed as being meant "The oligonucleotide of claim 8..."

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9210792
SA 68315

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/04/93. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9106626	16-05-91	AU-A- 7148191	31-05-91
WO-A- 9118997	12-12-91	AU-A- 7998491	31-12-91
		EP-A- 0531436	17-03-93

EPO FORM 10479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82